SP4 Gene targeting for Schizophrenia phenotype rescue in mice

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Abstract

Background: Schizophrenia is a complex psychiatric disorder affecting millions worldwide, with symptoms spanning cognitive deficits to hallucinations. Previous research has identified the SP4 gene as a significant risk factor for schizophrenia, associated with severe disruptions in neuronal function. Gene therapy presents a promising approach to addressing the genetic underpinnings of schizophrenia directly. This study aims to validate the SP4 gene as a drug target, as well as to explore the possibility to genetically rescue schizophrenic phenotypes. **Methods:** This study employed the adeno-associated virus (AAV) vector AAV-PHP.eB to deliver the eSYN-iCre construct across the entire brain in adult mice, leading to re-expression of the Sp4 gene. Schizophrenic phenotypes determined in previous studies were tested such as prepulse inhibition (PPI), response to ketamine and contextual memory. **Results:** The AAV-mediated gene therapy showed partial success in rescuing the PPI deficits in mice and significantly mitigated ketamine-induced hyperlocomotion in male mice, though results in female mice were complicated due to increase of locomotion caused by the Cre. Significant memory deficits were shown in not ameliorated by the treatment. **Discussion & Conclusion:** The study underscores the potential of the SP4 gene as a target for both gene therapy and small molecule drugs in treating schizophrenia. The successful delivery and expression of therapeutic genes across the brain demonstrate the viability of AAV vectors for CNS therapies, suggesting that gene therapy could offer a long-term remedy for psychiatric disorders. However, the gender-specific responses and partial rescue of phenotypes highlight the complexity of schizophrenia's genetic landscape and the need for further research into more targeted and comprehensive therapeutic approaches. This research opens avenues for the development of novel treatments that could profoundly impact the management and understanding of schizophrenia.

Introduction

Schizophrenia is a profound and problematic mental disorder, affects approximately 24 million people worldwide, according to the World Health Organization, with around 100.000 new cases emerging annually.[1] This condition is characterized by a diverse spectrum of symptoms, broadly categorized into positive symptoms (such as hallucinations and delusions), negative symptoms (including social withdrawal and anhedonia), and cognitive symptoms, which impact memory and decision-making. These manifestations significantly hinder an individual's ability to function and engage in daily life. Currently, therapeutic treatments for schizophrenia primarily target symptom management rather than addressing the underlying etiology of the disorder. Antipsychotic medications are at the forefront of treatment, aiming to alleviate positive symptoms by targeting neurotransmitter systems. However, these drugs often fall short in addressing negative and cognitive symptoms and can lead to substantial side effects, which can be debilitating. Additional treatments, such as psychotherapy, social support, and rehabilitation strategies, offer tools for managing the disorder, yet focus too much on mitigating symptoms rather than the root cause. Gene therapy is a promising new approach that could help treat schizophrenia by fixing or changing the genes and brain pathways that cause the disease. This method aims to address the problem at its source, potentially providing more effective and lasting treatments by not just reducing symptoms but also changing the disorder's progression.

In this study, we aim to explore whether gene therapy can treat schizophrenia, including its challenges and potential benefits. By examining the genetic factors behind the disease, we hope to find targeted strategies that address its root causes.

Background

Recent genetic screening studies, notably the Schizophrenia Exome Sequencing Meta-Analysis (SCHEMA)[2] and Genome-Wide Association Studies (GWAS)[3], have identified the SP4 gene as a significant risk factor for schizophrenia. These studies have shown that truncations in the SP4 gene are associated with a notably high odds ratio of 9.37 (95% CI: 3.38–29.7) for the development of schizophrenia, indicating a link between SP4 gene disruptions and the disease. The interactions between various schizophrenia-risk genes and their impact on the disease's pathogenesis remain poorly understood. To explore this, the role of SP4 as a transcription factor, specifically its interaction with GC-boxes, was investigated. GC-boxes are short DNA sequences that are rich in guanine (G) and cytosine (C) bases. These sequences are important regulatory elements in the promoter regions of many genes. GC-boxes are typically characterized by consensus sequences such as 5'-GGGCGG-3' (or variants thereof), and they play a crucial role in transcription regulation by binding specific transcription factors, notably the SP1 family of transcription factors, which includes SP4.[4]

An analysis was conducted to examine the prevalence of GC-boxes within the promoter regions of known schizophrenia-risk genes. The results indicated a significant overrepresentation of GC-boxes in these genes compared to what would be expected by chance. This finding suggests that SP4 may serve as a central regulatory hub, influencing the expression of multiple schizophrenia-risk genes through its interaction with GC-boxes. This mechanism could be a critical factor in the pathogenesis of schizophrenia, offering a potential explanation for how genetic predispositions to the disease are regulated at a molecular level. [4]

Earlier studies identified deficit in prepulse inhibition (PPI), ketamine hypersensitivity in locomotion, and memory deficit in Sp4 hypomorphic mice, establishing them as endophenotypes for schizophrenia. [5] Building on this, recent research showed that expression of Sp4 in forebrain GABAergic inhibitory neurons regulates behaviors like ketamine hypersensitivity using a Cre-LoxP system to restore the Sp4 expression. [6,7] The Cre-LoxP system was employed to selectively manipulate gene expression in specific neuronal populations of Sp4 hypomorphic mice. This genetic system uses Cre

recombinase, an enzyme that recognizes and recombines DNA at loxP sites—short DNA sequences inserted into the genome. By breeding mice that express Cre recombinase under neuron-specific promoters (such as Emx1 for forebrain excitatory neurons and Dlx5/6 for forebrain GABAergic neurons) with Sp4 hypomorphic mice, Cre recombinase was selectively expressed in targeted neurons. In these neurons, Cre recombinase excised the LacZ reporter gene flanked by loxP sites. This excision reactivated Sp4 expression specifically in the desired neurons, allowing restoration of Sp4 function in the neurons to be observed and the resultant changes in behavioral phenotypes. In figure 1, a schematic view of this technique is shown, as well as the LacZ staining of the respective brains.
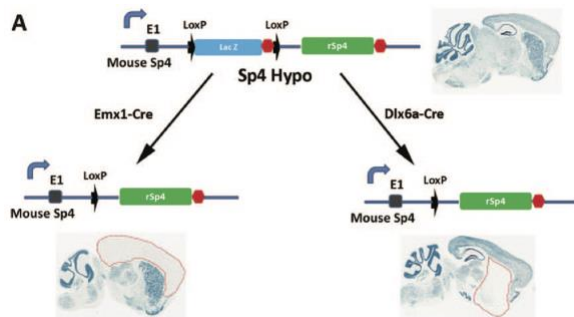

Figure 1, Figure 1, visualization of the LoxP/Cre system used in previous studies, with a target on excitatory and inhibitory neurons as shown on the left and right respectively.[6]

In contrast to this earlier study where Sp4 was restored in a specific neuronal cell type during embryogenesis, we propose to use the adeno-associated virus (AAV) vector AAV-PHP.eB to restore Sp4 gene in adult mice when phenotypes are developed. Such studies are critical for the development of therapeutics for schizophrenia patients. Unlike the previous Cre transgene approach, AAV-PHP.eB enables direct, widespread delivery of genetic modifications across the brain due to its ability to cross the blood-brain barrier. In the previous study, a F1 generation of mice crossed between Black Swissmice and the S129 mice was used. In this study, S129 crossed with C57 mice is used because of the compatibility of this strain with the AAV-PHP.eB to cross the blood brain barrier.

The AAV used in this study carries the eSYN-iCre construct, which combines the Synapsin promoter with a CMV enhancer to drive high levels of Cre recombinase expression specifically in neuronal cells. By injecting this AAV via mouse tail vein, Cre recombinase is expressed throughout the brain, allowing for the excision of the LacZ gene and subsequent activation of the Sp4 gene in all affected neurons. This approach aims to provide a comprehensive understanding of the SP4 gene's role across various brain regions in adult mice and its potential rescue effects on behavioral phenotypes associated with psychiatric disorders. [7]

This proof-of-concept study has two main objectives. The mice are treated at two months of age, corresponding to a more mature developmental stage akin to post-adolescence in humans. They were injected with gene therapy to simulate the real-life scenario, where schizophrenia is diagnosed often later in life. The primary aim of the research is to determine whether reintroducing the SP4 gene expression in the brain can rescue established schizophrenic phenotypes at this advanced stage. Furthermore, the effectiveness of the SP4 LoxP/Cre system is evaluated to validate the SP4 gene as a potential drug target for treating schizophrenia in human patients. This dual focus not only tests the feasibility of reversing schizophrenia symptoms with gene therapy but also assesses the utility of SP4 as a foundational element in the development of targeted therapeutic interventions.

Prepulse Inhibition (PPI)

Prepulse Inhibition (PPI) of the startle reflex is a widely used neurophysiological measure to assess sensory gating, a process that filters out unnecessary stimuli from higher cognitive processes. In schizophrenia, patients commonly exhibit reduced PPI, indicating a fundamental disruption in sensory gating mechanisms. In this study, using Sp4 hypomorphic mice, PPI serves as a crucial phenotype to model schizophrenia-related sensory gating deficits. By assessing the PPI responses in these genetically modified mice, the role of the SP4 gene in the neurobiological mechanisms of schizophrenia is further elucidated. Using this marker, the effectiveness of genetic therapy can be evaluated.

Ketamine Hypersensitivity

Ketamine, a known NMDAR (N-methyl-D-aspartate receptor) antagonist, is used in this research to investigate hypersensitivity reactions which mimic certain psychotic features observed in schizophrenia. In schizophrenia, altered NMDAR functions play a significant role in clinical phenotypes. Ketamine hypersensitivity tests the functionality of NMDAR pathways in Sp4 hypomorphic mice. These mice, due to their genetic alterations, will display an exaggerated response to ketamine or a less exaggerated response because of the therapy, serving as a model on how SP4 gene therapy would manifest to the rescue of schizophrenic phenotypes. [7]

Fear Conditioning

Fear conditioning is a behavioral paradigm used to study associative learning and memory, which are often impaired in schizophrenia. In this experiment, mice learn to associate a neutral conditioned stimulus (CS) with an aversive unconditioned stimulus (US), leading to a conditioned fear response, "freezing". This study utilizes both context and cued fear conditioning to examine the memory capabilities of Sp4 hypomorphic mice. The analysis of how these mice recall and respond to the fear-associated context and cues provide insight to the cognitive deficits associated with SP4 mutations and their relevance to similar cognitive impairments observed in schizophrenia.

Method

The Sp4 hypo mice were bred as described in [4] (Zhou et al. 2004). These mice were subsequently crossed with C57 mice to create a F2 generation of mice which contain the Cre-LoxP system. The mice were housed in a controlled temperature and humidity room, according to protocol. The mice were subject to a reversed day-night cycle, with daytime starting at 7.00 PM and nighttime at 7.00 AM. The mice had unrestricted access to food and water. They were weaned at three weeks old, due to the breeder mice being needed for the continued breeding for two more cohorts for another part of the over encompassing study.

A recombinant AAV-PHP.eB virus carrying Synapsin-Cre was used, which was intravenously injected into adult Sp4 hypo mice to restore Sp4 expression in the brain. This, in turn, rescued the schizophrenia-related phenotypes, namely deficient prepulse inhibition, deficient learning and memory, hypersensitivity to ketamine, as well as molecular and pathological abnormalities. These were examined 3 months after AAV injection to give the therapy time to take effect and for the hypothesized rescue in behavior and pathology to become apparent. [7]
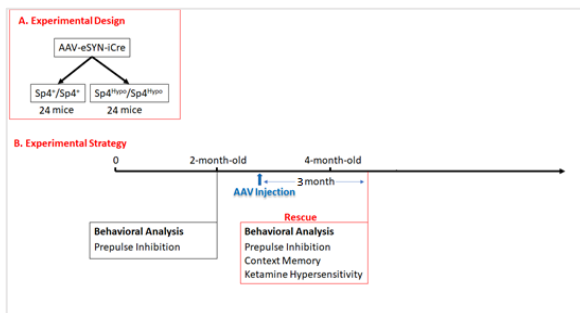

Figure 2, Experimental design and strategy for restoration of Sp4 expression. (A) 24 wildtype 12F/12M and 24 sibling Sp4 hypo mice 12F/12M will be bred from a generation of Sp4 heterozygous mice. (B) At an age of two months, the prepulse inhibition will be analysed. AAV injection at age 13 weeks. The second behavioral tests will be conducted 3 months after AAV injection.

Behavioral Analyses

Before each testing day, the mice would be brought up to the testing room an hour before testing for them to habituate to the new room and to recover from the way up. They were tested during the nighttime of their reversed cycle. The results were analyzed using Microsoft Excel, BPM, SPSS and R.

Mouse Startle

The prepulse inhibition (PPI) was measured in startle chambers (SR-LAB, San Diego Instruments) following the methodology of Ji et al. (2013). [8] The background noise level had 65 dB with the pulse being 40 ms for 120 dB. 80 ms before the pulse a prepulse of 69, 73 or 81 dB would be given, 4, 8 and 16 dB above the background noise. The prepulses should give increasing PPI, this way, if the mice

startle too high or too low, you could see in the results. The sessions started and ended with 5 "only-pulse" trials. In between, "no stimulus" (only background noise) and acoustic trials were done ten times in a pseudo-random order. PPI was calculated as a percentage of each acoustic prepulse trial: %PPI = 100% × {1 - [(Startle response for "prepulse + pulse")/(Startle response for "pulse-alone")]}. Between each session the boxes were wiped clean.

Video-Tracking Locomotion

Locomotor activity was measured using the Video-Tracker (VT) system as previously described (Ji et al., 2013). After acclimating to the testroom for 60 min, the mice were placed into one of four white plastic enclosures (41×41×34cm3). A camera mounted 158 cm above, generated a video signal for the Polytrack digitizer (San Diego Instruments). Each animal's position (x, y; in pixels) was sampled with a frequency of 18.18 Hz, which generated a coordinate file (x, y, t) which contained the location and the time spent there. The distance traveled was measured, as well as where the mice stayed most. The mice were placed in the enclosures for 30 minutes to acclimate to the enclosure, after which they received a ketamine injection as described below and were placed back in their enclosures for another 60min. Between each session, the enclosures were wiped clean to remove odors from previous mice. [7]

Ketamine

Ketamine was dissolved in saline and administered i.p. at a volume of 5ml/kg after 30 min habituation in the VT arena (Brody et al., 2003). The doses of ketamine were determined from the same F1 genetic background mouse in previous studies (Ji et al., 2013). A within-subjects crossover design was used for drug studies, with 2 weeks between drug treatments. [7,9]

Freeze monitor

Four fear conditioning shock chambers (26 x 22 x 18cm) made of metal walls with a clear plexiglass front and a metal grid floor were used in a 2 x 2 array (Med Associates Inc., East Fairfield, VT, USA). The chambers were put inside a monitoring box containing a video camera. The camera was connected to a video based digital recording system which also analyzed the freezing behavior (FreezeFrame, Actimetrics, St Evanston, IL, USA). After 3 minutes the conditioned stimulus tone of 85 dB started at 2800 Hz for 20 seconds, after which the unconditioned stimulus was given as a foot shock of 0.50 mA for 2 seconds. The freezing time was recorded during the conditioned stimulus, and during the post shock period. Three shocks were given with 1 minute spacing in between. The time spent freezing was recorded as the mice stood completely still.
After 24 hours, the context was tested. The mice were placed in the chamber and the freezing time was recorded for 16 minutes, in blocks of 2 minutes. The freezing component percentage was calculated by the system and compared between the groups.
At 48 hours, the cued freezing was tested. The chambers were modified to present a different environmental

context (eg shape, odor, light changes, floor type, background noise). Again, the first 3 minutes were recorded, after which 52 CS presentations were done. These presentations should give insight into how fast the Sp4 hypo mice learn no US is given after the CS, compared to the WT mice.[7]
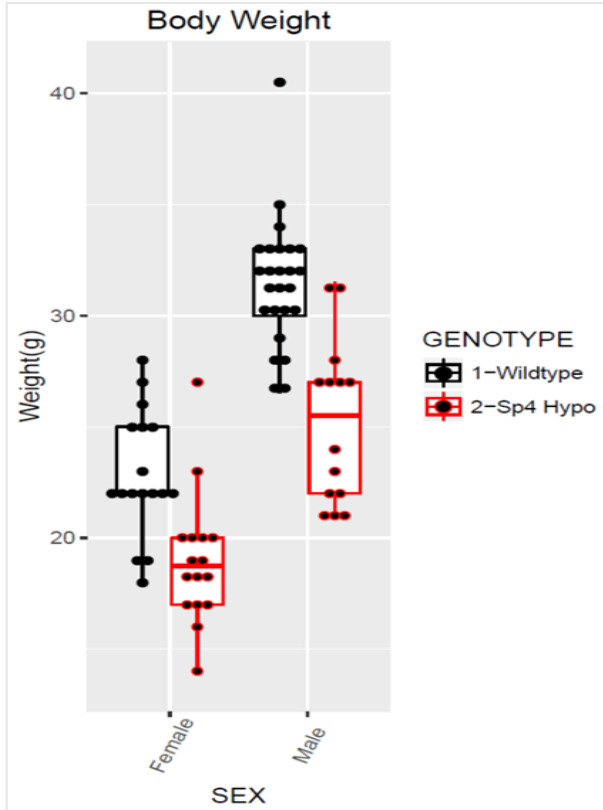
Results

Weight

Figure 3, Boxplot graph of the body weight distribution male and female mice 2-3 months after birth

Figure 3 presents the distribution of body weights for both male and female mice at two months of age, categorized by genotype: wildtype (WT) and Sp4 hypomorphic (Sp4 Hypo). The boxplots indicate that both male and female wildtype mice generally have higher body weights compared to their Sp4 Hypo counterparts. Specifically, the median body weight of male wildtype mice is markedly higher than that of male Sp4 Hypo mice. Similarly, female wildtype mice also exhibit a trend towards higher body weights than female Sp4 Hypo mice, though the difference appears less pronounced than in males.

Table 1, Two-way ANOVA of the weight distribution on sex and genotype

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
SEX	1	1211.6	1211.6	131.188	< 2e-16	***
GENOTYPE	1	458.1	458.1	49.600	1.19e-09	***
SEX:GENOTYPE	1	24.5	24.5	2.652	0.108	
Residuals	68	628.0	9.2			

Statistical analysis using a two-way ANOVA was performed to assess the effects of sex (male vs. female), genotype (wildtype vs. Sp4 Hypo), and their interaction on body weight. The analysis shows significant effects of both sex ($F_{(1, 68)}$ = 131.188, $p < 0.0001$) and genotype ($F_{(1, 68)}$ = 49.600, $p < 0.00001$) on body weight, as seen in table 1. However, the interaction between sex and genotype was not statistically significant ($F_{(1, 68)}$ = 2.652, $p = 0.108$).
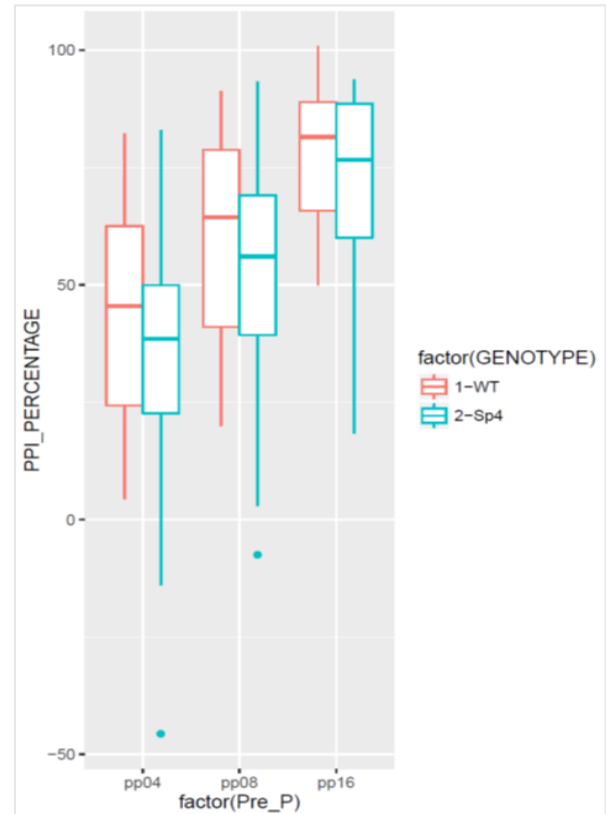
Mouse startle

Figure 4, Male-female combined PPI percentage per prepulse strength of 4, 8 and 16 dB above background noise

Figure 4 shows the prepulse inhibition percentage for the male and female mice combined against the three different prepulses used, at two months old.
A trend is seen for an increase in prepulse inhibition by a louder prepulse. As well as a trend comparing the wildtype to the Sp4 hypomorphs, but no significant difference, as shown in table 2.

Table 2, Two-way ANOVA of the PPI for sex and genotype

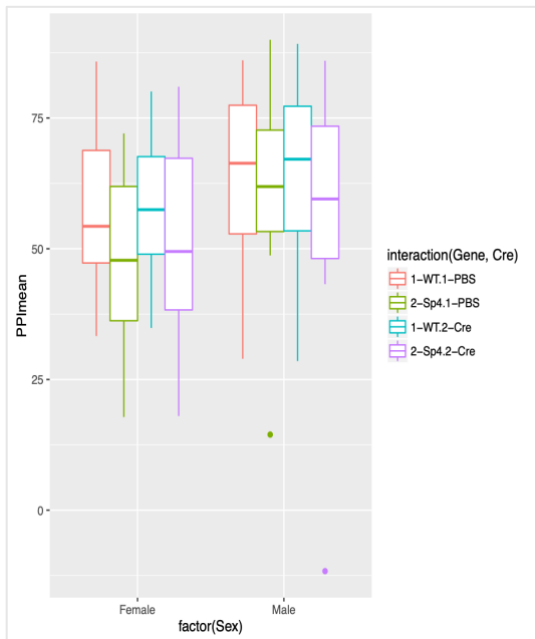|	Df	Sum Sq	Mean Sq	F value	Pr(>F)
sex	1	2682	2682	2.133	0.149
geno	1	2988	2988	2.376	0.128
sex:geno	1	140	140	0.111	0.740
Residuals	68	85512	1258		

Figure 5, Mean PPI per group at 2 months old balanced in groups

Figure 5 illustrates the distribution of mean PPI scores calculated across prepulse levels pp04, pp08, and pp16, after being balanced for sex and genotype, before treatment with either Cre recombinase or PBS. Balancing the groups is critical as it ensures that any differences observed post-treatment can be attributed solely to the effects of the treatment rather than underlying variations in sensory gating capacity among the animals. Balancing for sex and genotype prior to treatment helps control for inherent biological differences that could potentially confound the results.

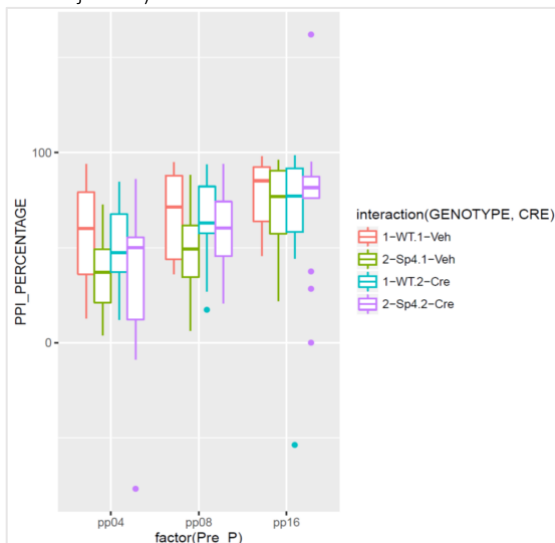AAV-eSYN-Cre Mouse Cohort (Rescue, 3 months after Virus Injection)

Figure 6, PPI distribution per prepulse strength, 3 months after AAV-eSYN-Cre injection

Figure 6 illustrates the PPI percentages for mice in the AAV-eSYN-Cre cohort, measured three months following virus injection. The cohort is divided into four groups based on genotype (wildtype, WT and Sp4 hypomorphic, Sp4) and treatment (vehicle, Veh and Cre recombinase, Cre). The PPI levels tested were pp04, pp08, and pp16.

Table 3, Multifactorial ANOVA of the PPI percentage of the mice 3 months after injection

```
               Df Sum Sq Mean Sq F value Pr(>F)
sex             1   3981    3981   2.643 0.1095
geno            1   4346    4346   2.885 0.0948 .
cre             1    364     364   0.242 0.6248
sex:geno        1    240     240   0.159 0.6914
sex:cre         1     28      28   0.019 0.8917
geno:cre        1    938     938   0.623 0.4332
sex:geno:cre    1   5771    5771   3.831 0.0551 .
Residuals      58  87373    1506
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Error: id:intensity
                     Df Sum Sq Mean Sq F value   Pr(>F)
intensity             2  26606   13303  46.316 1.64e-15 ***
sex:intensity         2    682     341   1.187    0.309
geno:intensity        2   2976    1488   5.181    0.007 **
cre:intensity         2    503     251   0.875    0.420
sex:geno:intensity    2    894     447   1.556    0.215
sex:cre:intensity     2    563     282   0.981    0.378
geno:cre:intensity    2    100      50   0.174    0.841
sex:geno:cre:intensity 2   1169     584   2.035    0.135
Residuals           116  33317     287
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

The results of a multifactorial ANOVA as shown in table 3 show significant interactions and main effects. The interaction between genotype and Cre recombinase treatment approached significance (p = 0.0551) and the genotype intensity analysis were found to be significant (p = 0.007).
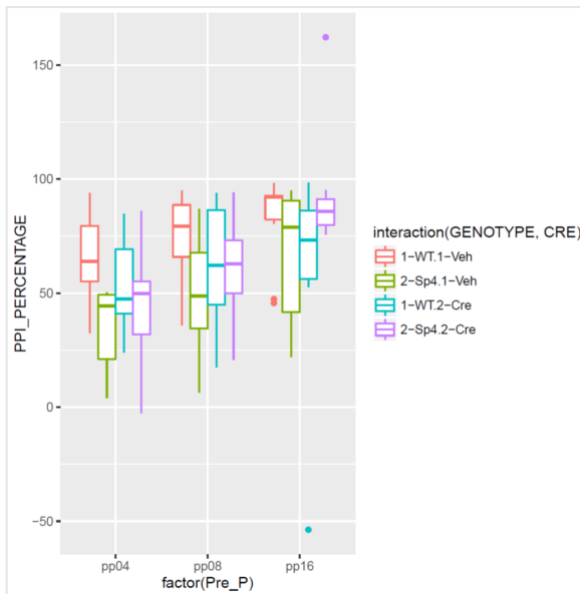
Male mice

Figure 7, PPI distribution in male mice 3 months after injection

Figure 7 presents the distribution of Prepulse Inhibition (PPI) percentages in male mice across three different prepulse levels: pp04, pp08, and pp16, categorized by genotype and treatment, three months after the injection.

Table 4, Multifactoral ANOVA of the PPI in male mice, 3 months after injection

```
            Df Sum Sq Mean Sq F value Pr(>F)
GENOTYPE     1   1288    1288   0.899 0.3507
CRE          1    318     318   0.222 0.6408
GENOTYPE:CRE 1   5730    5730   3.999 0.0546 .
Residuals   30  42980    1433
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' '

Error: ID:Pre_P
               Df Sum Sq Mean Sq F value   Pr(>F)
Pre_P           2  10173    5087  15.143 4.74e-06 ***
GENOTYPE:Pre_P  2   3532    1766   5.257  0.00787 **
CRE:Pre_P       2     22      11   0.033  0.96713
GENOTYPE:CRE:Pre_P 2  825     412   1.228  0.30022
Residuals      60  20155     336
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' '
```

As shown in table 4, statistical analysis using a two-way ANOVA was performed to assess the effects of genotype, treatment, and their interaction on PPI percentages. The analysis reveals that while the main effects of genotype and Cre recombinase treatment alone did not reach statistical significance, their interaction effect approached significance (F(1, 30) = 3.999, p = 0.0546).
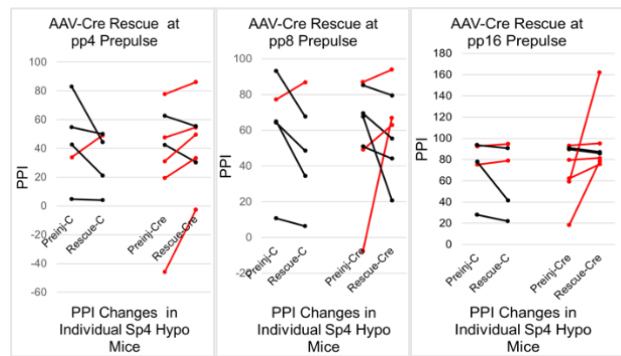

Figure 8, Individual PPI of Sp4 mice before and after injection, at each prepulse strength, red indicating an increase in PPI, black a decrease

This figure 8 presents the changes in Prepulse Inhibition (PPI) in individual Sp4 Hypo mice, illustrating the effect of AAV-Cre treatment at three different prepulse levels: pp4, pp8, and pp16. Each panel shows PPI values before and after the administration of AAV-Cre, enabling direct comparison of pre- and post-treatment responses within the same animals. Red lines indicating an increase in PPI and black indicating a decrease. The controls have an increase in PPI in 1, 1 and 2 mice, at pp4, pp8 and pp16 respectively. The Sp4 mice have 5, 3 and 5 mice whose PPI increased respectively.

Using the paired T-test, a trend in PPI improvement was found at pp4 and pp16 with a P value < 0.1.
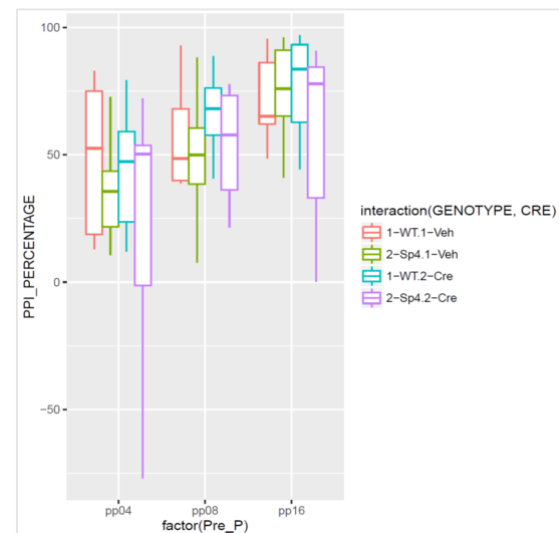
Female mice

Figure 9, PPI distribution in female mice at each prepulse strength, 3 months after injection

Figure 9 presents the distribution of Prepulse Inhibition percentages in female mice across three different prepulse levels: pp04, pp08, and pp16, categorized by genotype and treatment condition. The boxplots indicate that there are variations in PPI percentages across the different groups, but the differences are not substantial.

Table 5, Multifactorial ANOVA of the PPI in female mice, 3 months after injection

```
            Df Sum Sq Mean Sq F value  Pr(>F)
GENOTYPE     1   3274    3274   2.065   0.162
CRE          1     99      99   0.062   0.804
GENOTYPE:CRE 1    980     980   0.618   0.438
Residuals   28  44393    1585

Error: ID:Pre_P
              Df Sum Sq Mean Sq F value   Pr(>F)
Pre_P          2  17114    8557  36.407 7.42e-11 ***
GENOTYPE:Pre_P 2    339     170   0.721   0.491
CRE:Pre_P      2   1042     521   2.217   0.118
GENOTYPE:CRE:Pre_P 2  444     222   0.944   0.395
Residuals     56  13162     235
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

As shown in table 5, statistical analysis using a two-way ANOVA was performed to assess the effects of genotype, treatment, and their interaction on PPI percentages. The analysis shows that the main effects of genotype ($F_{(1, 28)}$ = 2.065, p = 0.162) and Cre recombinase treatment ($F_{(1, 28)}$ = 0.062, p = 0.804) were not statistically significant. Additionally, the interaction between genotype and treatment ($F_{(1, 28)}$ = 0.618, p = 0.438) was also not significant.

Ketamine hypersensitivity in video tracker open field
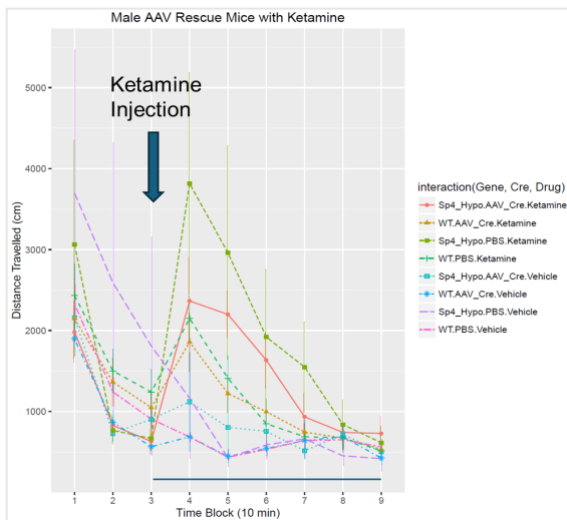
Male mice

Figure 10, Average distance traveled in each time block in open field for male mice, divided per genotype, treatment and drug

Figure 10 illustrates the response of male AAV rescue mice to ketamine injection, measured in terms of distance traveled in an open field video tracker. The graph compares various groups of mice based on their genotype, viral treatment and ketamine treatment. The line graph shows the distance traveled during nine consecutive time blocks, each representing 10 minutes, around the time of ketamine injection (indicated by the arrow).

The graph shows a peak in activity (distance traveled) immediately following ketamine injection for all groups, with varying intensities. Notably, Sp4 Hypo mice treated without Cre and with ketamine (top green line) exhibited the highest peak in activity.

Sp4 Hypo mice treated with Cre and ketamine (red line) also show increased activity post-injection, but to a lesser extent compared to Sp4 Hypo mice without viral treatment. The mice that received no ketamine displayed little to no increased activity after injection.

Table 6, Multifactorial ANOVA of the results of the videotracker experiment for male mice, 3 months after injection

```
            Df   Sum Sq   Mean Sq F value  Pr(>F)
Gene         1 12464854 12464854   5.196  0.0299 *
Cre          1   767471   767471   0.320  0.5758
Gene:Cre     1   675162   675162   0.281  0.5996
Residuals   30 71961526  2398718
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Error: ID:Block
             Df   Sum Sq Mean Sq F value  Pr(>F)
Block         5 49991599 9998320  27.532 < 2e-16 ***
Block:Gene    5  7472111 1494422   4.115 0.00157 **
Block:Cre     5  1864053  372811   1.027 0.40413
Block:Gene:Cre 5 1572217  314443   0.866 0.50564
Residuals   150 54473188  363155
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Error: Within
              Df   Sum Sq  Mean Sq F value   Pr(>F)
Drug           1 40420848 40420848 65.788 7.54e-14 ***
Block:Drug     5 31453331  6290666 10.239 1.19e-08 ***
Gene:Drug      1  6149458  6149458 10.009  0.00183 **
Cre:Drug       1  1436250  1436250  2.338  0.12804
Block:Gene:Drug 5 1879384   375877  0.612  0.69100
Block:Cre:Drug  5 1892838   378568  0.616  0.68765
Gene:Cre:Drug   1 2083032  2083032  3.390  0.06723 .
Block:Gene:Cre:Drug 5 648327 129665  0.211  0.95751
Residuals    180 110593247  614407
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

The multifactorial ANOVA for the ketamine hypersensitivity experiment for the male mice is shown in table 6. In the initial analysis of genotype, Cre, and their interaction shows only significant effects for genotype (Gene: p=0.0299, Cre: p=0.5758, Gene:Cre: p=0.5996). However, further analysis focusing on the interaction between genotype, drug, and time block (within-group analysis) revealed significant interactions. The interaction between Gene:Cre:Drug is almost significant (p=0.06723) highlighted with a red box in the table.
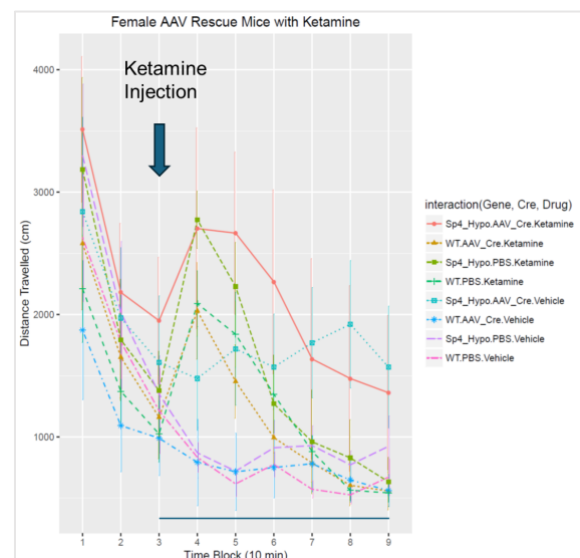
Female mice

Figure 11, Average distance traveled in each time block in open field for female mice, divided per genotype, treatment and drug

Figure 11 illustrates the response of female AAV rescue mice to ketamine injection, measured in terms of distance traveled in an open field video tracker. The graph compares various groups of mice based on their genotype, viral treatment and ketamine treatment. Like the previous figure the distance traveled is divided into 9 timeblocks of 10 minutes and a ketamine or saline injection at t=3.

All groups injected with ketamine exhibit a peak in locomotor activity following the injection, with varying degrees of intensity and duration. The control groups did not show such behavior.

Table 7, Multifactoral ANOVA of the results of the videotracker experiment for male mice, 3 months after injection

```
            Df    Sum Sq  Mean Sq F value  Pr(>F)
Gene         1  29863787 29863787  5.760  0.023 *
Cre          1   8528449  8528449  1.645  0.210
Gene:Cre     1  11218430 11218430  2.164  0.152
Residuals   29 150353016  5184587
---
Signif. codes:  0 ë***í 0.001 ë**í 0.01 ë*í 0.05 ë.í 0.1 ë í 1

Error: ID:Block
                Df    Sum Sq Mean Sq F value   Pr(>F)
Block            5  39365817 7873163  20.222  ***
Block:Gene       5    399636   79927   0.205   0.960
Block:Cre        5    929425  185885   0.477   0.793
Block:Gene:Cre   5   1431670  286334   0.735   0.598
Residuals      145  56453674  389336
---
Signif. codes:  0 ë***í 0.001 ë**í 0.01 ë*í 0.05 ë.í 0.1 ë í 1

Error: Within
                  Df    Sum Sq  Mean Sq F value   Pr(>F)
Drug               1  24540142 24540142 27.055 5.51e-07 ***
Block:Drug         5  37053593  7410719  8.170 5.98e-07 ***
Gene:Drug          1     61802    61802  0.068  0.794
Cre:Drug           1    534490   534490  0.589  0.444
Block:Gene:Drug    5    836733   167347  0.184  0.968
Block:Cre:Drug     5    813909   162782  0.179  0.970
Gene:Cre:Drug      1       520      520  0.001  0.981
Block:Gene:Cre:Drug 5   1256881   251376  0.277  0.925
Residuals        174 157826560   907049
---
Signif. codes:  0 ë***í 0.001 ë**í 0.01 ë*í 0.05 ë.í 0.1 ë í 1
```

Table 7 shows the ANOVA analysis of the videotracker results for the female mice. There is a significant difference in locomotion between the ketamine and vehicle groups. There is no difference in peak hight between the Sp4_Hypo/AAV_Cre.Ketamine and the Sp4_Hypo/PBS.Ketamine group. However, the Sp4_Hypo/AAV_Cre.Vehicle group has an elevated base locomotion, and a peak less high in comparison to the untreated group, which complicates the analysis of these data.
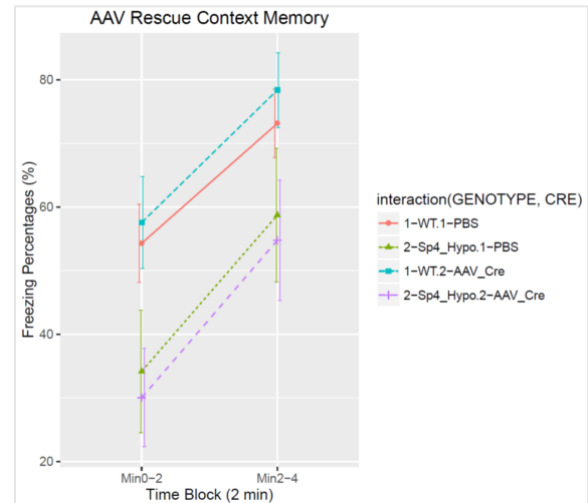
Fear conditioning

Figure 12, Percentage component freezing time per time block, male and female mice combined

Figure 12 displays the freezing percentage of both female and male mice across two time blocks (0–2 minutes and 2–4 minutes) after reintroduction to the context where they previously received shocks. The results show that WT mice exhibit consistently higher freezing percentages than Sp4 Hypo mice.

Table 8, Multifactoral ANOVA analysis of the fear conditioning test

```
                  Df Sum Sq Mean Sq F value  Pr(>F)
SEX                1      0       0   0.000 0.99662
GENOTYPE           1  15234   15234   9.009 0.00396 **
CRE                1     41      41   0.024 0.87639
SEX:GENOTYPE       1   1065    1065   0.630 0.43058
SEX:CRE            1    823     823   0.487 0.48827
GENOTYPE:CRE       1    217     217   0.128 0.72154
SEX:GENOTYPE:CRE   1      2       2   0.001 0.97338
Residuals         58  98076    1691
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Error: ID:TB
                  Df Sum Sq Mean Sq F value   Pr(>F)
TB                 1  15666   15666  77.330 2.91e-12 ***
SEX:TB             1    165     165   0.813  0.3709
GENOTYPE:TB        1    142     142   0.700  0.4061
CRE:TB             1     19      19   0.094  0.7603
SEX:GENOTYPE:TB    1    802     802   3.960  0.0513 .
SEX:CRE:TB         1     69      69   0.340  0.5622
GENOTYPE:CRE:TB    1     37      37   0.185  0.6687
SEX:GENOTYPE:CRE:TB 1   344     344   1.700  0.1975
Residuals         58  11750     203
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

The statistical analysis supports these observations, as shown in table 8, highlighting a significant effect of genotype on context memory retention, with WT mice outperforming Sp4 Hypo mice ($F_{(1, 29)} = 9.009$, p = 0.00396). However, the interaction between genotype and Cre treatment does not reach statistical significance ($F_{(1, 29)} = 0.128$, p = 0.72154), demonstrating that the AAV-Cre intervention fails to ameliorate the memory deficits observed in Sp4 Hypo mice. Additionally, no significant effects were found that directly attribute changes in memory performance to the Cre treatment alone, which implies the limited impact of this genetic intervention on enhancing context memory in the Sp4 mice.
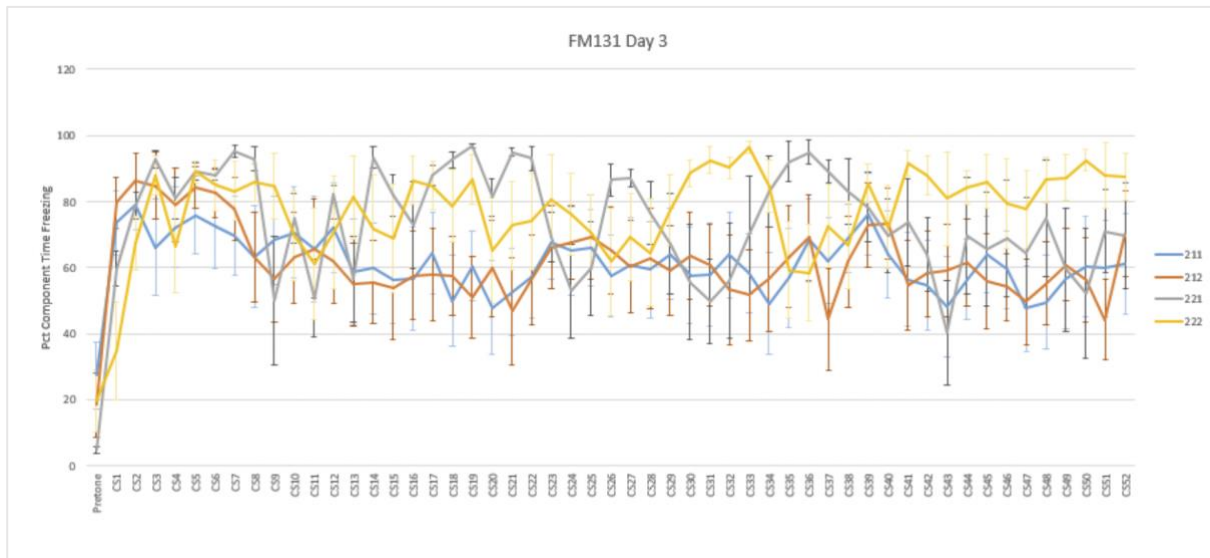
Figure 13, Percentage component time Freezing per cue on day 3 of the fear monitor test

Figure 13 shows the percentage freezing component time during the conditioned stimulus for the different male groups, with the mean standard error shown as brackets in the color of the corresponding line: WT, Vehicle (211) WT, Cre (212) Sp4, Vehicle (221), Sp4, Cre (222)
As shown in figure 13, the variance in the freezing time was too great to argue for any effect seen by the genotype or treatment. An overall decrease in freezing time is seen in the WT mice, implying a greater learning capability for them. However, during the experiment, the behavior of the mice was unpredictable, with some mice falling asleep, and others not responding to the cues at all.

Discussion

Weight
The bodyweight data of the mice as described in figure 1 reveal significant differences between the wildtype and Sp4 hypomorphic mice, with WT mice displaying higher body weights across both sexes. This difference between the WT mice and the Sp4 mice is statistically significant. Which implies there is an impact of the SP4 gene on physical development. The Sp4 hypomorphic mutation leads to a reduction in body weight at two months of age in both sexes, with a more profound effect observed in males. The absence of a significant interaction between sex and genotype (p = 0.108) suggests that the underlying mechanisms affecting body weight may operate similarly in male and female mice.

Prepulse inhibition
In terms of sensory gating, measured by PPI, trend differences are observed between the WT and Sp4 Hypo mice at two months old. This suggests that the expected difference had not yet completely manifested due to the mice's age or developmental stage. A trend in PPI difference was seen in the effect of PPI volume, this

confirms the prepulse volume was loud enough and not too loud for the startle.

The tests performed after the introduction of AAV-Cre on the whole group, showed a significant difference between the WT mice and the Sp4 Hypo mice, indicating that the genotype did influence the PPI, and that it had not yet completely manifested at 2 months old.
When looking at the effect of the AAV-Cre treatment, the results had to split between the males and the females. In the females, no significant difference was seen between the genotypes or the treated groups, however, the mice in these groups were so small that the variability between measurements was high, probably causing the lack of significance. The males almost show a significant difference when looking at the groups, however looking at an individual level, increase in prepulse inhibition is presented. Which is a positive clue to the AAV-cre treatment being effective.

Ketamine hypersensitivity
Additionally, the ketamine hypersensitivity tests, conducted to explore the behavioral response to pharmacological stimuli, reveal that Sp4 Hypo mice exhibit increased locomotor activity in response to ketamine, particularly those not treated with Cre. The results of the male mice show a significant effect of the genotype and ketamine. The male Sp4 mice show significant increase in locomotion compared to the WT mice. This implies the genetic altering of the mice works, which corresponds to a model for testing ketamine hypersensitivity, a schizophrenic phenotype. The interaction between genotype and Cre almost reached significance. This is a positive clue for that the LoxP/Cre AAV technique worked, as also seen in the male mice in the PPI test.
The insignificant difference in the female group does not conclude that the Cre worked, however, because of the increased locomotion of the Sp4.Cre Vehicle group. A suggestion can be made that Cre causes an increase in locomotion in the females, and that, the spike caused by

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the ketamine is lower in the treated sp4 group than in the untreated group.

Contextual memory

The results of the Freeze monitor test show a significant increase in freezing time by the WT mice compared to the Sp4 hypo mice, indicating a better memory of context p = 0.00396. Both WT and Sp4 Hypo groups show an increase in freezing behavior from the first to the second time block, suggesting that the memory recall strengthens with longer exposure to the context. Sp4 Hypo mice, whether treated with PBS or AAV-Cre, do not show significant differences in their freezing behavior, indicating that the AAV-Cre treatment does not enhance memory recall in these genetically modified mice. The cued stimulus test on day three lead to no meaningful results. Which underscores the complexity of genetic influences on behavior like fear, memory and learning capabilities and the challenges gene therapy targeting neuropsychiatric disorders face.

Limitations

Looking at the limitations of this study, a few things need to be addressed. The Sp4 mice were not bred optimally, they had to be bred in a rush because other cohorts of this type of mice needed to be bred as well. This caused them to be weaned while they were not yet fully developed. They were below their weight target, and some of them had malformations in their spines or hunchbacks. Also, because the use of F2 generation mice brings its limitations because of the genetic segregation, a F1 generation would be preferable. Due to the cost of running large cohorts, the groups were small. This combined with the low weight and suboptimal development of the mice caused for more variability which may have caused some trends not to be significant.

Conclusion

This proof-of-concept study aimed to find a way to genetically rescue schizophrenia related phenotypes in adult mice. As seen in previous studies, the PPI and memory deficits in Sp4 hypo mice were present. Clues to the rescue of these phenotypes were seen as they almost reached significance. The ketamine hypersensitivity was present in the Sp4 hypo mice and the rescue of this hypersensitivity was hinted at in the males. These findings indicate a promising future for the technique of using the AAV-PHP.eB vector to deliver gene modifications across the brain, demonstrating a promising method for targeting the genetic cause of schizophrenia. By confirming the SP4 gene as a viable target for both gene therapy and small molecule drugs, this study opens the road to deeper investigation on this gene and its pathways, potentially leading to a cure for schizophrenia in the future.

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