# Metabotropic Glutamate receptor 2 and Dopamine receptor 2 gene expression predict sensorimotor gating response in the genetically heterogeneous NIH-HS rat strain

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## Abstract (150-250 words)

Disruption of sensorimotor gating causes “flooding” of irrelevant sensory input and is considered a congenital trait in several neurodevelopmental disorders. Prepulse inhibition of acoustic startle response (PPI) is the operational measurement and has a high translational validity. Pharmacological studies in rodents have linked alterations in serotonin, dopamine and glutamate signalling to PPI disruption. How PPI response is associated with gene expression levels of these receptors is unknown. PPI response was assessed in thirty-nine genetically heterogeneous NIH-Heterogeneous Stock rats and animals classified as High, Medium or Low PPI respondents. Expression levels of glutamate metabotropic receptor 2 (*Grm2*), dopamine receptor D2 (*Drd2)*, dopamine receptor D1 (*Drd1*), serotonin receptor 1A (*Htr1a*), serotonin receptor 2A (*Htr2a*) and homer scaffolding protein 1 (*Homer1*) were investigated in prefrontal cortex (PFC) and striatum (STR). When comparing the two extreme phenotypes, only *Drd2* in STR showed increased expression in the Low PPI group. A multinominal model fitting all genes and all groups indicated that *Grm2* in PFC, and *Grm2* and *Drd2* in the STR predicted PPI group. This was corroborated by a linear relationship of *Grm2* with PPI in PFC, and *Drd2* with PPI in STR. An interaction between gene region H3K27 methylation levels and PPI phenotype was observed for *Drd2* in STR. Gene enrichment analysis on an expression microarray dataset on Lewis rats confirmed enrichment of *Drd2* in PFC in relation to PPI. These findings contribute in the understanding of the genetic substrate behind alterations in sensorimotor gating, relevant for its linkage to neurodevelopmental disorders.

## Keywords

Sensorimotor gating response, schizophrenia, neurotransmitter receptors, postsynapse, gene expression, epigenetics

## Introduction

Sensorimotor gating, also known as pre-attentive filtering process, is an essential mechanism used in regulating and filtering out unwanted/unnecessary sensory inputs. Gating deficiency causes "flooding" of sensory stimuli [1] and is observed in relation to neuropsychiatric [2-4] and neurological diseases [5,6]. Moreover, sensorimotor gating deficiency is a highly heritable behavioural endophenotype and considered a risk indicator for schizophrenia spectrum disorders [7,8].

Prepulse inhibition (PPI) of acoustic startle response is the operational measure of sensorimotor gating. PPI response evaluates the reduction (inhibition) of an induced response (startle) after repeated exposure of acoustic pulses and pre-pulses [9,10]. PPI test was first intended for investigating inhibitory processes related to sensory flooding in patients with schizophrenia [11]. Indeed, people with schizophrenia, across race, show a decrease in PPI response compared to controls [2,12-15]. With time it has become clear that decreased PPI response is not exclusive for schizophrenia and is also observed in other disorders characterized by faulty inhibitory control response [10,16]. Nevertheless, PPI response is still very much used as a behavioural endophenotype for studying the underlying neurobiology behind schizophrenia. As this test has a high cross-species validity, which means that observations are easily translatable from animal to humans, PPI is a well-established test for determining the face validity of animal models of schizophrenia [17].

One of these animal models presenting a schizophrenia-like phenotype, and thereby also disrupted PPI, is the Roman High-Avoidance (RHA) rat strain [18-23]. When compared to its counterpart, the Roman Low-Avoidance (RLA) rat strain, the RHA strain moreover exhibits alterations in function, regulation and expression of schizophrenia-related receptors, such as serotonin receptor 1A (5-HT1AR), serotonin receptor 2A (5-HT2AR), Dopamine receptor D2 (D2R), and Metabotropic glutamate receptor 2 (mGluR2) [20,23-25]. Some of these alterations are directly linked to epigenetic modifications by trimethylation of histone H3 at lysine 27 (H3K27me3) at proximal promoter regions [24]. Pharmacological studies have implicated these receptors in the modulation of the PPI response [26-28], but to what extent differential gene expression of these receptors account for the different PPI response, and thereby alteration of gene expression directly can be related to disrupted PPI has not been investigated yet.

Here we made use of naïve genetically heterogeneous National Institutes of Health-Heterogeneous Stock (NIH-HS) rats, which represent a stock of rats as genetically heterogeneous as possible [29].

To obtain this stock, eight parental inbred strains were crossed, generating a high genetic recombination pattern and phenotypic variability more similar to human population than most commonly used laboratory rat strains. They constitute thereby an excellent tool to study the neurobiological and genetic basis of normal and thereby also abnormal (illness-related) complex traits [30,31]. The NIH-HS rats present a broad-spectrum of PPI responses comparable to the RLA strain PPI response [18]. Our aim was to investigate gene expression profiles of receptors and genes of interest and see how they associate with the normal variation in the PPI response.

Based on our previous observations, and on the relevance of these targets in relation to schizophrenia [20,23-25,32], we focused on the dopamine D2 (*Drd2*), serotonin 1A (*Htr1a*), serotonin 2A (*Htr2a*), and metabotropic glutamate 2 (*Grm2*) receptor genes and added dopamine D1 (*Drd1*) receptor and the postsynaptic scaffolding protein Homer1 (*Homer1*) to the study. We looked in prefrontal cortex (PFC) and striatum (STR), as these are areas included in the PPI brain circuitry [16,33]. We also looked at whether gene expression profiles were epigenetically modulated by either histone acetylation (H3ac) or histone methylation (H3K27me3). Lastly, we wanted to test in a dataset available through the GEO website, containing microarray expression profiling data from prefrontal cortex of inbred Lewis rats tested for PPI, whether gene sets related to synapse regulation/activation were enriched in relation to the PPI phenotype. For that we applied the Broad Institute Gene Set Enrichment Analysis (GSEA) and Leading Edge Analysis software [34].

## Methods and material

### Animals

A total of 39 male National Institute of Health – Heterogenous Stock (NIH-HS) rats were used for this study. The rat stock is genetically heterogeneous and is derived from an outcross breeding strategy of eight inbred rat strains (MR/N,WN/N,WKY/N,M520/N,F344/N,ACI/N,BN/SsN, and BUF/N strains) [29] from the permanent colonies maintained at our laboratory (Medical Psychology Unit, Dept. Psychiatry, and Forensic Medicine, School of Medicine, Autonomous University of Barcelona) since 2004. The NIH-HS rats used for this experiment were submitted to a PPI testing session following the procedure described elsewhere ([18] see below). The NIH-HS rats were housed in same-sexed pairs under standard conditions (12:12 hours light/dark cycle; 22±2oC; 50-70% humidity; food and water ad libitum) in standard (50 × 25 × 14 cm) macrolon cages. NIH-HS rats were approximately four months old of age (weight range 320-400 g) when the experimental assessment started. The PPI test was performed during light cycle.

### Prepulse inhibition of acoustic startle response

The NIH-HS rats were individually assessed in the sound-attenuated box (SR-Lab Startle Response System, San Diego Inst., San Diego, USA) as described by Oliveras et al. 2015[18] and Río-Álamos et al. 2019 [22]. Each rat was placed in a cylinder, in a box, which was placed on top of a platform with sensors detecting the rat's movement (startle).

The startle session included the following sound stimuli:

**1)** Habituation to the sound-attenuated box for 5 min
**2)** Background noise of 55dB + 10 single *pulse* of 105dB for 40ms.
**3)** 10 repeats of a block (set of 6 randomised trials [a-c])
  **a)** Background noise of 55dB
  **b)** Background noise of 55dB + *pulse* of 105dB for 40ms.
  **c)** Background noise of 55dB + *prepulse* of 65/70/75/80dB for 20ms + *pulse* of 105dB for 40ms.
**4)** 5 repeats of Background noise of 55dB + *pulse* of 105dB for 40ms.

Startle response was measured throughout the whole session. However, the measurements included in the equation for calculating the PPI response is the startle response after **3b** and **3c**. The degree of PPI was calculated in percentages for each *prepulse* (65/70/75/80dB), followed by determining the $\%PPI_{Total}$, which is the data used for the statistical analysis and will be referred to as PPI (response).

$$\%PPI = 100 - \frac{mean\ respond\ \mathbf{3c}}{mean\ respond\ \mathbf{3b}} \cdot 100 \quad \rightarrow \quad \%PPI_{Total} = \frac{\sum \%PPI}{4}$$

#### mRNA extraction and purification

Four weeks after the PPI evaluation, the NIH-HS rats were euthanised and PFC and STR extracted. For each region, two pieces of tissue were extracted. One was immediately frozen in liquid nitrogen and the other piece immediately processed for protein cross-linking in order to be used for the chromatin immunoprecipitation assay (see further down). Both pieces of tissue were kept at -80ºC until further use.

mRNA was extracted using the commercially available NucleoSpin® RNA/Protein kit (Macherey-Nagel; cat. no. 740933) following the manufactures protocol. 10 mg of tissue was homogenised by repeated pipetting in lysis buffer with added 1% β-mercaptoethanol. RNA samples were subsequently treated with the Turbo DNA-freeTM Kit (Ambion; cat. no. AM1907) according to the manufactures protocol to degrade the remaining DNA in the sample. mRNA concentration was determined using the Thermo Scientific™ NanoDrop 2000c spectrophotometer (Thermo Scientific™). RNA integrity number (RIN) was determined using the RNA nano chips (Agilent Technologies; cat. no. 5067-1511) and the 2100 Agilent Bioanalyzer (Agilent Technologies, Palo Alta, CA, USA). Only samples with an A260/A280 ratio ≥ 1.8 and a RIN value ≥ 5 were included for the further analysis.

#### Two-step reverse transcription real-time quantitative polymerase chain reaction

For the reverse transcription, qScriptTM cDNA SuperMix (5x) (Quata; cat. no. 95048) was used in accordance with the manufactures protocol. In short, 200ng of the purified mRNA samples and Universal Rat Reference RNA (Agilent Technologies; cat. no. 740200) were reverse transcribed into complementary DNA (cDNA). The reverse transcription mix was successively incubated at 25°C for 5min; 42°C for 30 min; 85°C for 5min; and finally kept at 4°C until the samples were stored at -20°C. The cDNA products were diluted 1:4 with of RNase/DNase-free water.

Each sample was run on a 96-well plate in duplicates and one reaction contained 1 µL diluted cDNA, appropriate concentrations of the primers (**Table 1**), RNase/DNase-free water and Fast SYBR® Green master mix (Applied Biosystems; cat. no. 4385612) according to the manufactures protocol. On each 96-well plate triplicates of RNase/DNase-free water were also included as negative control for contamination, as well as triplicates containing cDNA from Rat Universal Reference total RNA as a calibrator for intraplate variance. Each plate was run on the QuantStudio 3 Real-Time PCR

System (Applied Biosystems; cat. no. A28567), with the most optimal qPCR programs for each primer: *Grm2*, *Drd1*, *Drd2*, *Htr1a*, *Htr2a*, and *Homer1* worked at: 10min at 95°C; (15s at 95°C; 30s at 60°C) x40, and *Gapdh* and *Rpl13a* worked at: 10min at 95°C; (15s at 95°C; 30s at 60°C; 30s at 72°C) x40. Following the PCR program, a melting curve was generated with the program: 15s at 95°C; 15s at 55°C; 15s at 95°C. The melting curve was generated to verify that each sample only produced a single product. The relative gene expression was calculated by utilising the Normalised Relative Quantification equation from Pfaffl (2001), normalising to the two reference genes, *Gapdh* and *Rpl13a.*

**Protein Cross-Linking and Chromatin Immunoprecipitation**

Proteins were cross-linked to the DNA following the same protocol as described in Fomsgaard et al. 2017 [19]. Briefly, once dissected, the pieces of tissue were placed immediately in 10 mL PBS (pH 7.4) containing 1% formaldehyde and incubated for 20 min at room temperature (RT). The reaction was stopped by adding 0.125M glycine (Sigma; cat. no. G8898) and further incubated for 5 min at RT. The samples were the centrifuged at 1500×g for 5 min at 4°C. The supernatant was discarded, and the pellets resuspended in 1 mL cold PBS+0.1% Protease Inhibitor Cocktail (Sigma; cat. no. P8340) and transferred to an Eppendorf tube. Samples were centrifuged at 1500×g for 5 min at 4°C. The supernatant was discarded, and the pellets were kept at −80 °C until further use.

The chromatin immunoprecipitation was performed using the EZ-Magna ChIP™ A kit (Millipore; cat. no. 17-408) according to the manufactures protocol. The thawed and prepared samples were incubated over night at 4ºC with magnetic protein beads (from the kit) and one of the following primary antibodies: rabbit anti-acetyl histone H3 (Millipore; cat. no. 06-599B), rabbit anti-trimethyl histone H3 (Lys27) (Millipore; cat. no. 07-449), or rabbit IgG (Millipore; cat. no. PP64B). 20 µL of the prepared samples without antibodies were kept as input control. The following day, the protein-bound magnetic beads were isolated and washed, using the magnetic separator (Millipore; cat. no. 20-400) and Salt Immune Complex Wash Buffer (Millipore), respectively. The input control and immunoprecipitated samples were eluted using the ChIP elution buffer+1%proteinase K and incubated for 2h at 62°C, 10 min at 95°C and kept at RT in order to reverse crosslink the protein/DNA complex. The magnetic separator was used to remove the magnetic beads from the solution. The samples were transferred and collected in Spin Filters, from where the DNA was eluted. The eluted DNA was kept at −20°C until further use.

#### qPCR Analysis of Immunoprecipitated DNA

Each sample was run on a 384-well plate in quadruplicates. One reaction contained 2 µL concentration DNA, 200 nM primers (**Table 2**), and PowerUp™ SYBR® Green Master Mix (Thermo Fisher Scientific Inc.; cat. no. A25742). Each plate was run on the QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems; cat. no. 4485691) with the most optimal qPCR program: 2min at 50°C; 2min at 95°C; (15s at 95°C; 1 min at 60°C) x45. After the qPCR program was run a melting curve was generated with the program 15s at 95°C; 1min at 60°C; 15s at 95°C to verify that each sample only produced a single product. *Gapdh* expression at the promoter region of was used to validate the chromatin immunoprecipitation assay.

#### Microarray RNA expression dataset

We downloaded from the GEO website repository a microarray transcription profiling dataset from a study performed on inbred Lewis rats tested for PPI (GSE14903). The dataset contains expression data generated on an Affimetrix Rat Genome 230 2.0 array from PFC from 10 Lewis male rats, with their corresponding total PPI scores. Considering the 10 total PPI scores (median 26.02; range 35.73; max 42.52, min 6.7), we selected the 3 animals with the highest (median 40.19; range 3.14; max 42.52, min 39.38) and the lowest scores (median 10.73; range 11.59; max 18.38, min 6.79) and downloaded the microarray data from these 6 animals. For the GSEA analysis we selected all GO gene sets available through the Broad Institute Molecular Signatures Database (MSigDB) that were retrieved through the keyword "synapse". The output was 22 gene sets. We ran the GSEA analysis with these gene sets and selected the significant gene sets (FDR q-val<0.25) for the Leading Edge Analysis.

#### Statistical analysis

Before analysing the data, the ROUT method was run for each PPI group for identifying outliers. Outliers were excluded from further analysis. Kruskal-Wallis test with post-hoc Dunn's multiple comparison test was conducted for analysing the PPI response across the different groups. For the mRNA expression data, a D'Agostino & Pearson Omnibus normality test was run. Not all data followed a Gaussian distribution, and we therefore performed a multiple t-test with Holm-Sidak post-hoc correction, without assuming consistent standard deviation. A model was constructed by a multinomial logistic regression analysis with PPI as the nominal dependent variable and the genes of interest as independent variables. Models were constructed independently for each region. Missing values were excluded from the model and final sample inclusion can be seen in supplementary **Table 1s**. The genes with a significant contribution to the model were further analysed in a linear regression

analysis. Mann-Whitney U test was conducted to analyse H3ac and H3K27me3 binding levels at the proximal promoter region of *Gapdh*. Two-way repeated measure (mixed design) ANOVA with Šidák multiple comparison tests was conducted for analysing the H3ac and H3K27me3 across the different gene areas. All statistical analyses were performed using GraphPad Prism 7 and 8, and IBM SPSS Statistics 22.0. $p < 0.05$ was considered significant.

## Results

### Prepulse inhibition of acoustic startle response differs among groups

Column analysis was performed for the 39 rats based on their PPI response and accordingly distributed into three groups. Rats included in the 25th percentiles with the lowest PPI response (n = 10; min. value: 9.39; max. value: 47.80) and rats included in the 75th percentiles with the highest PPI response (n = 10; min. value: 71.46; max. value; 88.92), were selected into respectively a Low PPI and High PPI group. The remaining rats (n = 19; min. value: 48.71; max. value: 70.71) were included in the Medium PPI group. When comparing the PPI response, there was a significantly different PPI response, for the exposure to the prepulse stimuli of 65 dB (H = 28.36, $p$ <0.0001), 70 dB (H = 22.20, $p$ <0.0001), 75 dB (H = 18.82, $p$ <0.0001), 80 dB (H = 16.69, $p$ = 0.0002), and for the total PPI (H = 32.35, $p$ <0.0001), across the three groups. Additionally, a significant difference between the Low PPI and High PPI was observed when performing Dunn's multiple comparisons test for the prepulse stimuli of 65 dB ($p$ <0.0001), 70 dB ($p$ <0.0001), 75 dB ($p$ <0.0001), 80 dB ($p$ = 0.0001), and for the total PPI ($p$ <0.0001) (**Table 3**).

### Increased gene expression in the Low PPI Group

When comparing the relative mRNA expression between the PPI groups for the PFC (**Fig 1a**), a statistic significant increase of *Grm2* (t(15) = 2.292; $p$ = 0.0368), *Drd2* (t(16) = 2.384; $p$ = 0.0299), *Htr1a* (t(16) = 2.480; $p$ = 0.0246), and *Homer1* (t(15) = 2.848; $p$ = 0.0122) was observed in the Low PPI group compared to the High PPI group. However, these differences did not stay significant after correcting for multiple testing.

For the STR (**Fig 1b**), a statistic significant increase of the relative mRNA expression of *Drd2* (t(17) = 3.124; $p$ = 0.0062), *Htr1a* (t(18) = 2.294; $p$ = 0.0340), and *Htr2a* (t(18) = 2.266; $p$ = 0.0360) was observed in the Low PPI group compared to the High PPI group. Following correction for multiple testing, *Drd2* ($p$ = 0.0365) maintained its statistically significance.

### Model fitting gene expression with PPI response

A multinomial logistic regression analysis including all genes was performed for analysing to what extent overall gene expression predicted PPI response.

The model shows that overall gene expression levels significantly predict the classification of the animals in their corresponding PPI groups. This was so for both the PFC ($\chi^2$ = 27.937; df = 12; $p$ = 0.006) and STR ($\chi^2$ = 29.843; df = 12; $p$ = 0.003) (supplementary **Table 2s**). The classification accuracy for PFC is 75.0% and for STR is 72.7% (supplementary **Table 3s**). When testing each gene's

unique contribution to the model we found that, for the PFC, *Grm2* ($\chi^2$ = 8.239; df = 2; $p$ = 0.016) , and, for the STR, both *Grm2* ($\chi^2$ = 7.722; df = 2; $p$ = 0.021) and *Drd2* ($\chi^2$ = 15.829; df = 2; $p$ = 0.000) were the genes which contributed significantly to the model (**Table 4**).

#### Linear Relationship Between PPI Response and *Drd2* and *Grm2* expression

When analysing the linear regression of genes contributing to the model with the PPI response, we found a statistically significant linear relationship between PPI and *Grm2* expression levels in the PFC ($F_{(1,32)}$ = 9.5; $R^2$ = 0.23; $p$ = 0.0043) (**Fig 2a**) and *Drd2* expression levels in the STR ($F_{(1,33)}$ = 5.2; $R^2$ = 0.14; $p$ = 0.0286) (**Fig 2b**).

No statistically significant linear relationship was observed between PPI and *Grm2* ($F_{(1,36)}$= 2.3; $R^2$ = 0.06; $p$ = 0.1391) in the STR.

#### Differences in epigenetic modulation through H3K27me3

We wanted to further investigate if the observed differential gene expression levels accounting for the different PPI response were due to different epigenetic regulation. H3ac and H3K27me3 levels at the distal promoter (at -1.4kb of TSS), proximal promoter (up to 1 kb upstream of TSS), and TSS for *Grm2* in PFC, and *Drd2* in STR were analysed. Furthermore, H3ac and H3K27me3 at the proximal promoter region of *Gapdh* was used to validate the chromatin immunoprecipitation assay (**Fig 3a and 3b**). Here we found no significant difference in the binding of H3ac (U = 30, $p$ = 0.6058) or H3K27me3 (U = 10, $p$ = 0.4286) in the PFC between high PPI and Low PPI. Furthermore, we found no significant difference in the binding of H3ac (U = 33, $p$ = 0.2176) or H3K27me3 (U = 33, $p$ = 0.2103) in the STR between high PPI and Low PPI.

For the *Grm2 i*n the PFC, there was a significant difference in H3ac binding across the different gene areas ($F_{(2, 30)}$ = 71.65; $p$ <0.0001), but no gene area x PPI group interaction ($F_{(2, 30)}$ = 0.9390; $p$ = 0.4022) or overall effect of PPI phenotype ($F_{(1, 16)}$ = 0.003; $p$ = 0.9606). The same was observed for H3K27me3 binding, with a significant difference across the different gene areas ($F_{(2, 18)}$ = 101.1; $p$ <0.0001), and no and no gene area x PPI group interaction ($F_{(2, 18)}$ = 2.751; $p$ = 0.0907) or significant overall effect of PPI phenotype ($F_{(1, 9)}$ = 0.094; $p$ = 0.7658) (**Fig 3a**).

For *Drd2* in STR, H3ac binding across the different gene areas was significant different ($F_{(2, 33)}$ = 203.4; $p$ <0.0001), but no gene area x phenotype interaction ($F_{(2, 33)}$ = 0,1808; $p$ = 0.8354) or general effect of PPI phenotype ($F_{(1, 18)}$ = 1.256; $p$ = 0.2771). On the contrary, for the H3K27me3 binding, there was a significant difference across the different gene areas ($F_{(2, 35)}$ = 6.127; $p$ = 0.0052), with a

significant gene area x PPI interaction ($F_{(2, 35)} = 4.383$; $p = 0.02$). There was no significant overall effect of PPI phenotype ($F_{(1, 18)} = 0.5829$; $p = 0.4551$). (**Fig 3b**).

#### GSEA analysis shows enrichment of D2R expression in relation to PPI phenotype

From the 22 gene sets retrieved from the MSigDB repository, only 12 gene sets passed the threshold set by the GSEA software of a minimum of 15 coincident genes. From these gene sets, 5 were significantly enriched in the High PPI phenotype (FDR<25%) and 1 in the Low PPI phenotype (FDR<25%) (**Fig 4a**, Supplementary **file 1**). When running a Leading Edge Analysis, *DRD1* and *DRD2* were the two genes most represented in the enrichment score of the significant gene sets (**Fig 4b**).

## Discussion

The results of this study show for the first time how expression levels of genes associated with schizophrenia correlate and predict the PPI response. This was specially so for *Grm2* in PFC and *Drd2* in STR. Both gene products, mGluR2 and D2R, have been implicated in schizophrenia [35-40], as well as the cortico-striatal circuitry in PPI regulation [33,41,42]. Specially, the STR is an important regulatory centre for the neural circuitry involved in the PPI response [16]. Bilateral lesions of dorsomedial STR leads to a marked reduction in PPI [43].

There is a strong evidence from previous pharmacological studies of a regulatory effect of D2R activation on PPI response. D2R agonists decreases the PPI response [26,44], while D2R antagonists increases the PPI response [23,45-48]. Supporting our observations, a recent study showed higher basal protein levels of D2R in striatum of Low-PPI mice compared to High-PPI mice [49]. In regards of mGluR2, there is no such evidence of a direct regulatory role of the receptor on PPI response. The mGluR2 primarily functions as a presynaptic autoreceptor regulating glutamate release [50]. Therefore, activation of this receptor is expected to have an indirect modulatory effect. Indeed, mGluR2/3 agonist [27,51] and mGluR2 positive allosteric modulators (PAMs) [36,52] have been successfully used for reversing induced hyperglutamatergic PPI deficiency. Even more interesting, systemic administration of a mGluR2 agonist has been reported to regulate striatal dopamine release [53], suggesting an interaction between mGluR2 and D2R activation in the modulation of the PPI response. Absence of the mGluR2 may cause an imbalance in this interaction affecting the PPI response. This is what we may see in the RHA-I strain, where a stop-codon mutation in the *Grm2* results in zero transcription of this receptor [24] accompanied by a schizophrenia-like phenotype with highly disrupted PPI [18,22].

Most of the studies looking at the involvement of neurotransmitter systems in the PPI response are based on pharmacological challenges and/or experimentally induced PPI deficits [9]. The relevance of PPI, however, relies on its importance as a translational cognitive endophenotype of schizophrenia [54] as it has by now been well established that PPI is highly heritable, with a complex polygenic background [54-56]. Understanding the neurobiology behind naturally occurring variations in the PPI spectrum can therefore provide us with more accurate information on the genetic architecture behind this endophenotype. The use in this study of a special type of rats, the genetically heterogeneous (outbred) NIH-HS rat stock, allowed us to address to what extend PPI responses are associated with variations in expression levels of a specific set of genes of interest. Even though our most significant

results point to *Grm2* and *Drd2* we cannot exclude that increased expression of *Htr1a* and *Homer1*, in the PFC, and *Htr1a*, and *Htr2a*, in the STR, also participate in the low PPI response. A decrease in PPI response when rats are exposed to 5-HT1AR and 5-HT2AR agonists have been reported [9,27,28,57,58]. Our own studies also report increased binding of 5-HT1AR and 5-HT2AR in the prefrontal cortex of the RHA rat strain [20,24]. Indirectly, studies on Homer1a knock-out rodents report low PPI response in addition to other schizophrenia-like behaviours in these mice lacking Homer 1 expression [59,60]. As mentioned above, these observations are based on pharmacological studies or constructed animal models supporting the general idea that, not only one, but multiple neurotransmitter systems are involved in regulating this response, since neurotransmitters interact and modulate each other at different levels [9]. Our results do not contradict this but point to a substantial interaction between *Dr2d* and *Gmr2* gene expression and behavioural phenotype.

To investigate to what extend this differential expression could originate from differences in epigenetic modulation of these two genes we looked for acetylation of histone 3 (H3ac) and trimethylation levels of lysine 27 on histone 3 (H3K27me3). We focused on these modifications as they are involved in gene regulation during embryotic development [61-63] and we have previously found to be involved in the regulation of mGluR2 and 5-HT2AR expression [24,35,64]. We found an interaction between PPI groups and gene region H3K27me3 levels for the Drd2 in the STR, with the Low PPI group showing lower methylation levels at the promoter region. Histone lysine methylation is implicated in gene silencing, acting as a “dial” or “switch” of gene expression by fine-tuning expression levels from active to poised to inactive [65]. This corresponds well with the higher *Drd2* expression in the Low PPI group. Results are though marginal and need to be further corroborated in further studies. We cannot exclude either that other gene sites or histone modifications apply, as we did not look for them. Nevertheless, these observations are in line with a plausible epigenetic programming of genes involved in specific behavioural manifestations [66,67], which is important as epigenetic modifications are reversible and open up for the possibility of adjusting pathological behaviours by pharmacologically targeting them.

Finally, we corroborated overrepresentation of *Drd2* in relation to PPI phenotype by running a GSEA analysis on a publicly available microarray dataset for PFC on Lewis rats tested for PPI. This method derives its power by focusing, instead of on single genes, on gene sets, that are defined based on groups of genes that share common biological functions [34]. This way we could determine that gene sets related with synaptic function where enriched in relation to a divergent PPI response. Gene set

enrichment indicates that the probability for a group of genes to be randomly differentially regulated in one group versus the other is low, independently of the direction of expression. GSEA is considered primarily an explorative tool. The fact that *Drd1* also was overrepresented in this analysis suggest this receptor to be a target of interest for further study, even though we did not see a differential expression in our own dataset. Altered D1R signalling has also been related to PPI deficits [68].

In conclusion, we present strong evidence supporting that differential expression of *Grm2* in the PFC, and *Drd2* in STR predict the PPI response. Striatal *Drd2* expression seems to be epigenetically modulated through H3K27me3 at the gene promoter region, suggesting a dynamic regulation that can be an adaptation to other pathways differentially regulated but not investigated. Enhanced *Grm2* expression could indicate compensatory effects towards glutamatergic tonus. Our results add further support to the idea of dopaminergic and glutamatergic signalling playing a central role in the cortico-striatal modulation of PPI response, and thereby the sensorimotor gating response.

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## Compliance with ethical standards

Experiments were performed in accordance with the Spanish legislation on "Protection of Animals Used for Experimental and Other Scientific Purposes" and the European Communities Council Directive (2010/63/EU) on this subject. Every effort was made to minimize any suffering of the animals used in this study.

## Disclosure of potential conflicts of interests

There is no conflict of interest to declare.

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## Figures

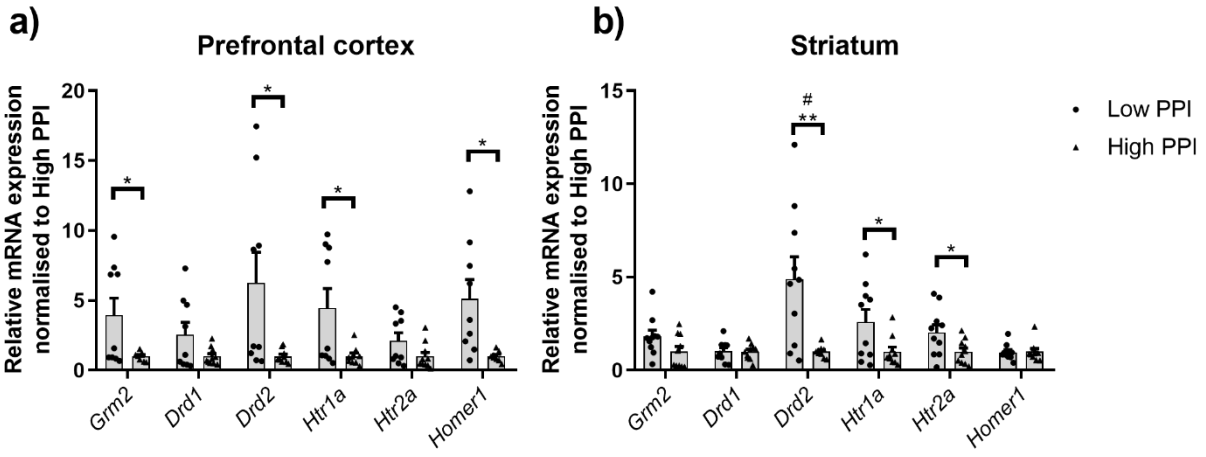



**Fig 1** Bar chart illustrating means and individual values for the relative mRNA expression of genes of interest between Low PPI group (25$^{th}$ percentiles of total PPI) and High PPI group (75$^{th}$ percentiles of total PPI). **a)** A significant increased expression of *Grm2*, *Drd2*, *Htr1a*, and *Homer1* was observed in the Low PPI group in the prefrontal cortex. Differences did not pass multiple testing correction. **b)** A significant increased expression of *Drd2*, *Htr1a*, and *Htr2a* was observed in the Low PPI group in the striatum. *Drd2* passed multiple testing correction. Data is presented as mean ± SEM. Statistical significance was set at <0.05. *$p \leq 0.05$, ** $p \leq 0.01$, $^{\#}p \leq 0.05$ (after Holm-Sidak post-hoc correction)

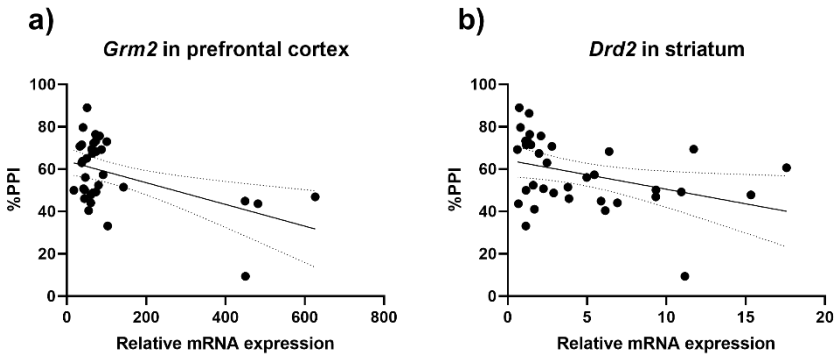



**Fig 2** Linear regression analysis of the specific PPI response as the dependent variable, and the mRNA expression as the independent variable. **a)** *Grm2* expression levels in the PFC. $R^2 = 0.23$; $p = 0.0043$. **b)** *Drd2* expression levels in the STR. $R^2 = 0.14$; $p = 0.0286$. Statistical significance was set at <0.05

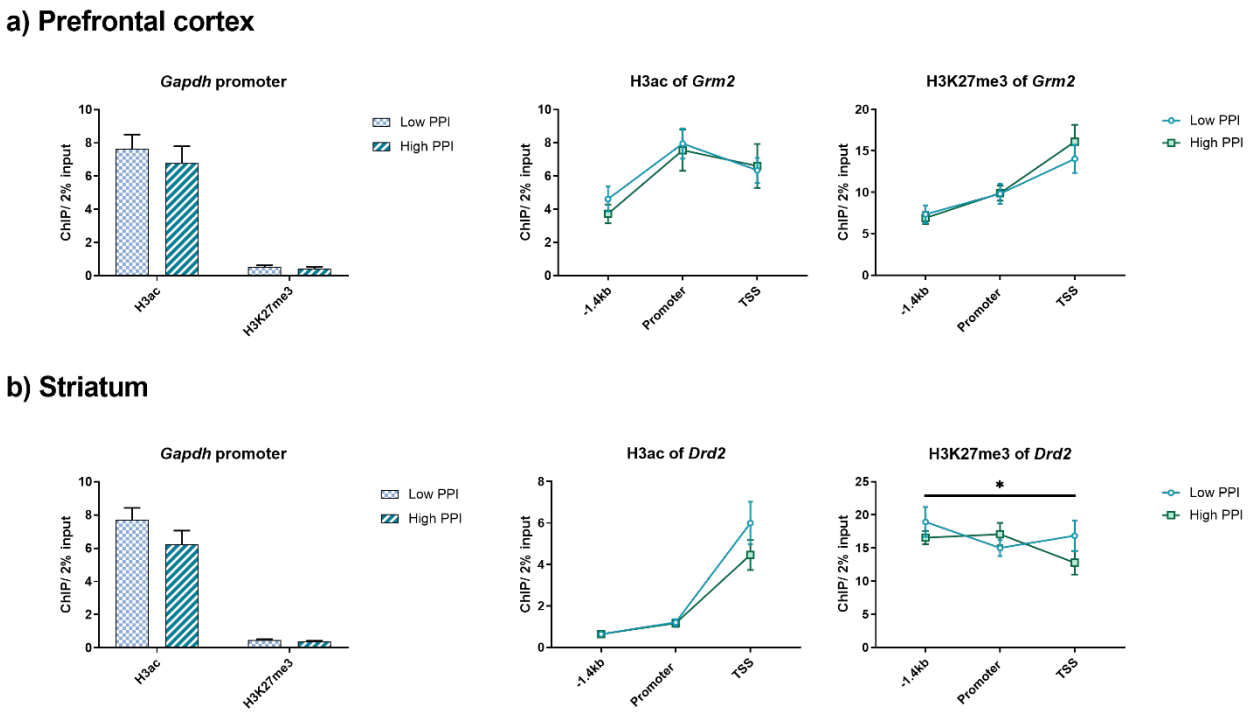
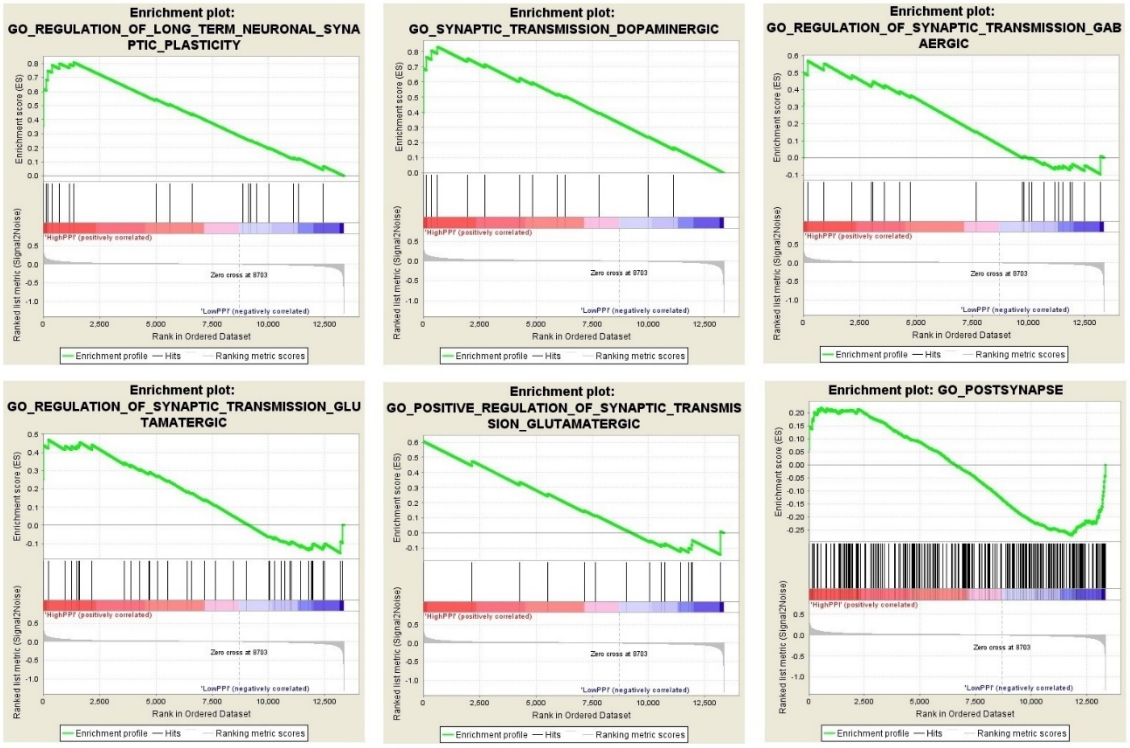



**Fig 3** Comparison of acetylation of histone H3 (H3ac) and trimethylation of histone H3 at lysine 27 (H3K27me3) levels, across the distal promoter (-1.4kb), proximal promoter (promoter), and transcriptional start site (TSS) for genes of interest in the Low PPI group and the High PPI group. H3ac and H3K27me3 at the promoter region of *Gapdh* was used to validate the chromatin immunoprecipitation assay. **a)** In the prefrontal cortex, Two-way repeated measure (mixed design) ANOVA with Šidák multiple comparison tests showed statistically significant difference in binding of H3ac and H3K27me3 for the different gene areas for *Grm2* and *Drd2*, but no significant group effect or gene area x group interaction. **b)** In the striatum, Two-way repeated measure (mixed design) ANOVA with Šidák multiple comparison tests showed significant difference in binding of H3ac and H3K27me3 for the different gene areas of the *Drd2*, but no significant group effect. A significant gene area x group interaction for H3K27me3 binding was observed. Data is presented as mean ± SEM. Statistical significance was set at <0.05. $*p \leq 0.05$

a




| Gene Set | Number of genes | Enrichment Score (ES) | Nominal (ES) | NOM p-val | FDR q-val | FWER p-val |
|---|---|---|---|---|---|---|
| GO_REGULATION_OF_LONG_TERM_NEURONAL_SYNAPTIC_PLASTICITY | 19 | 0.8078495 | 21.524.754 | 0.0 | 0.0 | 0.0 |
| GO_SYNAPTIC_TRANSMISSION_DOPAMINERGIC | 15 | 0.83325726 | 20.681.047 | 0.0 | 4,38E-01 | 0.001 |
| GO_REGULATION_OF_SYNAPTIC_TRANSMISSION_GABAERGIC | 24 | 0.5683552 | 15.663.054 | 0.033043478 | 0.05720024 | 0.157 |
| GO_POSITIVE_REGULATION_OF_SYNAPTIC_TRANSMISSION_GLUTAMATERGIC | 15 | 0.60600334 | 15.343.316 | 0.041050903 | 0.050630726 | 0.182 |
| GO_REGULATION_OF_SYNAPTIC_TRANSMISSION_GLUTAMATERGIC | 43 | 0.4691117 | 14.746.102 | 0.04194631 | 0.06474092 | 0.275 |
| GO_POSTSYNAPSE | 271 | -0.2722237 | -11.839.176 | 0.11519608 | 0.22051929 | 0.686 |

b

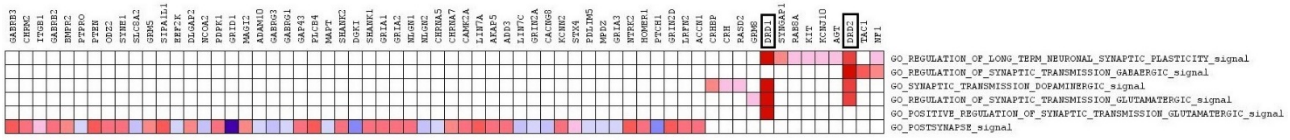



**Fig 4** Gene Set Enrichment Analysis (GSEA) **a)** Enrichment plots of the gene sets significantly enriched in relation to the High PPI phenotype (ES>1) or the Low PPI phenotype (ES<1) after running a GSEA analysis on the GSE14903 microarray dataset. Gene set name, number of genes in the set, enrichment score (ES), nominal enrichment score, Nominal p value (NOM p-val), False Discovery Rate (FDR q-val) and Family Wise Error Rate (FWER p-val) are given in the table. **b)** Results of the Leading-Edge Analysis, showing an overlap for the presence of the *DRD2* gene, also of the *DRD1*, in the core enrichment for the significant gene sets

## Tables

**Table 1** Listed the primers sequences used, their product size, and primer concentration for the Two-step qPCR

| Target | Sequence (5' → 3') | Product size | Concentration |
|---|---|---|---|
| *Gapdh*<br>NM_017008.4 | F: CATCAAGAAGGTGGTGAAGCA<br>R: CTGTTGAAGTCACAGGAGACA | 93 bp | 300 nM |
| *Rpl13a*<br>NM_173340.2 | F: AGCAGCTCTTGAGGCTAAGG<br>R: GGGTTCACACCAAGAGTCCA | 102 bp | 300 nM |
| *Grm2*<br>NM_001105711.1 | F: GTGGTGACATTGCGCTGTAA<br>R: GCGATGAGGAGCACATTGTA | 74 bp | 500 nM |
| *Drd1*<br>NM_012546.2 | F: GGAGGACACCGAGGATGA<br>R: ATGAGGGACGATGAAATGG | 69 bp | 500 nM |
| *Drd2*<br>NM_012547.1 | F: TCGAGCTTTCAGAGCCAACC<br>R: GGGTACAGTTGCCCTTGAGTG | 50 bp | 300 nM |
| *Htr1a*<br>NM_012585.1 | F: CCAAGAAGAGCCTGAACGGA<br>R: CTGCCTCACTGCCCCATTAG | 101 bp | 300 nM |
| *Htr2a*<br>NM_017254.1 | F: CCGCTTCAACTCCAGAACCA<br>R: GATTGGCATGGATATACCTACAGA | 79 bp | 500 nM |
| *Homer1*<br>AJ276327.1 | F: CACCCGATGTGACACAGAACTC<br>R: TGATTGCTGAATTGAATGTGTACCT | 93 bp | 300 nM |

**Table 2** Listed the primers used for analysing the chromatin immunoprecipitated (ChIP) products and their product size.

| Gene | Target | Sequence (5' → 3') | Product size |
|---|---|---|---|
| *Gapdh* NC_005103.4 | Proximal promoter | F: AACCCTCATCCGGTCACTTCC<br>R: CGAGTAGCTGGGCCTCTCTCA | 149 bp |
| *Grm2* NC_005107.4 | Distal promoter | F: GGCAGAGCTGGATCTGGAAG<br>R: AATGGGAGACAAGGTGGCAG | 92 bp |
| | Proximal promoter | F: ATTCAGCACCACAAGGTGGACA<br>R: CAATTTGGCCTGCACCTCTCGC | 132 bp |
| | Transcriptional start site | F: ATGAGCACCGAGGCATACAG<br>R: GATGCGGTCCAGTGCAAAAA | 56 bp |
| *Drd2* NC_005107.4 | Distal promoter | F: ACATCTACAACTGGCAAGGGA<br>R: GTTTTCCACCCAGTCGTGTG | 52 bp |
| | Proximal promoter | F: AGTGCTTCAGCTAGCCCTTG<br>R: GGGGAAGGAACCTTGAGAGC | 206 bp |
| | Transcriptional start site | F: TGTACAAGGGGCGGGGTT<br>R: CACAAGAGGGGACCAGCC | 122 bp |

**Table 3** Overview of the rats prepulse inhibition of acoustic startle response (PPI) to different prepulse intensities. The "Total" is the average %PPI score for the different prepulse intensities. * indicate significantly different PPI response across the three groups. # indicate significant difference between the Low and High of PPI groups following post-hoc analysis. Data is presented as mean ± SEM. Kruskal-Wallis test with post-hoc Dunn's multiple comparison test have been performed and Statistical significance was set at <0.05. *** $p \leq 0.001$, **** <0.0001, $^{\#\#\#}$ $p \leq 0.001$, $^{\#\#\#\#}$ <0.0001.

| | Low PPI (n = 10) | Medium PPI (n = 19) | High PPI (n = 10) |
|---|---|---|---|
| **%PPI 65dB ****** | 8.66 ± 10.57 $^{\#\#\#\#}$ | 42.55 ± 2.81 | 70.94 ± 3.01 |
| **%PPI 70dB ****** | 41.48 ± 4.38 $^{\#\#\#\#}$ | 53.63 ± 2.90 | 76.07 ± 2.52 |
| **%PPI 75dB ****** | 51.07 ± 3.30 $^{\#\#\#\#}$ | 66.55 ± 2.13 | 77.36 ± 2.73 |
| **%PPI 80dB ***** | 57.71 ± 4.77 $^{\#\#\#}$ | 73.20 ± 3.15 | 82.91 ± 1.52 |
| **%PPI$_{Total}$ ****** | 39.73 ± 3.63 $^{\#\#\#\#}$ | 59.98 ± 1.82 | 76.82 ± 1.98 |

**Table 4** Model predicting PPI response based on Multinomial Logistic Regression analysis of expression levels for the individual genes of interest. For the prefrontal cortex, *Grm2* contributes significantly to the model. For the striatum, *Grm2* and *Drd2* contribute significantly to the model. Statistical significance was set at <0.05. *$p \leq 0.05$, *** $p \leq 0.001$

| | | **Model Fitting Criteria** | **Likelihood Ratio Tests** | | |
|---|---|---|---|---|---|
| **Brain region** | **Effect** | -2 Log Likelihood of Reduced Model | Chi-Square | df | Sig. |
| Prefrontal cortex | *Grm2* | 48.047 | 8.239 | 2 | ***0.016**** |
| | *Htr1a* | 42.722 | 2.915 | 2 | 0.233 |
| | *Htr2a* | 40.288 | 0.481 | 2 | 0.786 |
| | *Drd1* | 44.292 | 4.485 | 2 | 0.106 |
| | *Drd2* | 43.291 | 3.483 | 2 | 0.175 |
| | *Homer1* | 42.288 | 2.480 | 2 | 0.289 |
| Striatum | *Grm2* | 49.154 | 7.722 | 2 | ***0.021**** |
| | *Htr1a* | 46.294 | 4.862 | 2 | 0.088 |
| | *Htr2a* | 41.917 | 0.486 | 2 | 0.784 |
| | *Drd1* | 45.545 | 4.113 | 2 | 0.128 |
| | *Drd2* | 57.260 | 15.829 | 2 | ***0.000**** |
| | *Homer1* | 45.519 | 4.087 | 2 | 0.130 |

## Supplementary material

**Table 1s** Sample distribution for performing the Multinomial Logistic Regression analysis of the relative mRNA expression of genes of interest (GOI) in the regions of interest. Originally, the Low PPI group included the rats within the 25$^{th}$ percentiles of the PPI response (n = 10), the High PPI group included the rats within the 75$^{th}$ percentiles PPI response (n = 10), and the Medium PPI group included the rats between the two extreme response groups (n = 19). However, after outliers were identified and excluded using the ROUT method with a 1% false discovery rate for each PPI group, for each GOI.

| Brain region | Low PPI | Medium PPI | High PPI |
|---|---|---|---|
| Prefrontal cortex | 9 | 15 | 8 |
| Striatum | 10 | 14 | 9 |

**Table 2s** Model Fitting Information based on Multinomial Logistic Regression analysis of the relative mRNA expression of genes of interest in the regions of interest. The models produced can with statistical significance predict the PPI group (Low PPI, Medium PPI, High PPI) in the prefrontal cortex and striatum according to overall gene expression levels. Statistical significance set at <0.05. ** $p \leq 0.01$

| Brain region | Model Fitting Criteria | Likelihood Ratio Tests | | |
|---|---|---|---|---|
| | -2 Log Likelihood | Chi-Square | df | Sig. |
| Prefrontal cortex | 39.807 | 27.937 | 12 | ***0.006**** |
| Striatum | 41.432 | 29.843 | 12 | ***0.003**** |

**Table 3s** Classification table illustrating the classification into the correct PPI group according to the Multinomial Logistic Regression analysis model.

| Brain region | Observed | Predicted based on model | | | Percent Correct |
|---|---|---|---|---|---|
| | | Low | Medium | High | |
| Prefrontal cortex | Low | 4 | 4 | 1 | 44.4% |
| | Medium | 0 | 14 | 1 | 93.3% |
| | High | 0 | 2 | 6 | 75.0% |
| | Overall Percentage | 12.5% | 62.5% | 25.0% | **75.0%** |
| Striatum | Low | 6 | 4 | 0 | 60.0% |
| | Medium | 2 | 10 | 2 | 71.4% |
| | High | 0 | 1 | 8 | 88.9% |
| | Overall Percentage | 24.2% | 45.5% | 30.3% | **72.7%** |