CHAPTER 5

Studying social group behaviours in Pcdh9 deficient mice using the Visible Burrow System

Maria Bove\textsuperscript{1,2}, Kevin Ike\textsuperscript{1}, Betty E. Hornix\textsuperscript{1}, Adriaan Eldering\textsuperscript{1}, Maria Grazia Morgese\textsuperscript{3}, Stefania Schiavone\textsuperscript{3}, Luigia Trabace\textsuperscript{3}, Martien J. H. Kas\textsuperscript{1}

\textsuperscript{1}Groningen Institute for Evolutionary Life Science, University of Groningen, The Netherlands
\textsuperscript{2}Department of Physiology and Pharmacology "V. Erspamer", "Sapienza" University of Rome, Italy
\textsuperscript{3}Department of Clinical and Experimental Medicine, University of Foggia, Foggia, Italy

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Abstract

Genetic deletion of genes related to neuropsychiatric disorders allow us to study gene function in relation to disease features. In this regard, genetic ablation of Pcdh9 gene has recently been associated to sensory processing deficits and impaired social interactions, that may be relevant for symptomatology across the neuropsychiatric spectrum. To study social behavioural phenotypes under semi-natural conditions, we have recently implemented the Visible Burrow System (VBS), a highly social habitat that may provide useful insights in group-housed social dynamics in a translational way. Recently, we have shown the suitability of the VBS to investigate social withdrawal features and their underlying neurobiology in a mouse model for ASD-like behavioural phenotypes. In this study we investigated for the first time mixed-genotype colonies in the VBS. In particular, we evaluated sociability and social withdrawal features in Pcdh9 Wild Type (WT), Heterozygous (HET) and Homozygous (HOM) gene knockout (KO) mice, housed together in VBS. In light of the known sensory deficits in this genetic mouse model, we also investigated GABAergic and glutamatergic alterations in the somatosensory cortex of Pcdh9 WT, KO HET and KO HOM mice. In this preliminary study, our results reported no differences in terms of social behaviours and non-social behaviours among the three genotypes. Moreover, no genotype differences were detected in GABA content in somatosensory cortex, for both standard housing condition and VBS colony housing. Interestingly, glutamate levels were significantly increased in Pcdh9 KO HOM mice housed in standard cages, while this increase was not found in HOM Pcdh9-deficient mice housed in the VBS, suggesting a beneficial effect of this highly social environment on glutamate increase induced by Pcdh9 genetic ablation. In conclusion the VBS, beyond its employment as a tool to assess sociability in a translational way, might be further used as a behavioural paradigm to test pharmacological treatments aiming at restoring social dysfunctions commonly occurring in several neuropsychiatric disorders, such as social withdrawal.
5.1 Introduction

Mental disorders are defined and classified in the Diagnostic and Statistical Manual of Mental Disorders (DSM–5), edited by the American Psychiatric Association (APA), in order to improve diagnoses, treatments and researches (www.apa.org, APA website). The burden of mental disorders is continuously growing with significant impacts on health in all countries of the world (www.apa.org, APA website). During the last decades, diagnosis in psychiatry only focused on subjective symptoms and observable signs (Goodkind et al., 2015). Although symptoms are an important starting point, genetics and neurobiology underlying these symptoms need to be deeply investigated. Interestingly, genetic and clinical analyses found similarities across a wide variety of diagnoses, suggesting that a common neurobiological substrate across mental illnesses might exist (Goodkind et al., 2015). In addition, genetic analyses have identified common polymorphisms associated with a large range of neuropsychiatric diseases and comorbidity among them is considerably higher than expected (Brugger & Howes, 2017). Moreover, several mental diseases, such as schizophrenia, Autism Spectrum Disorders (ASD), Alzheimer’s disease, anxiety disorders and major depression diseases, share a number of common symptoms, especially regarding the social sphere (Goodkind et al., 2015).

In this context, disrupted sociability and consequent social withdrawal are recently receiving great attention as important symptoms that deeply affect the quality of life in neuropsychiatric patients. However, the pathophysiology underlying these symptoms still need to be fully elucidated and current treatments are not able to improve these relevant behavioural alterations (Wilson & Koenig, 2014).

Furthermore, rodents can be a helpful tool to study behavioural alterations related to neuropsychiatric diseases and their underlying neurobiology and genetics. In this regard, to assess behavioural alterations in a translational way, habitats mimicking the natural environment in which group-housed dynamics can be deeply analyzed might be highly useful. In our study, we implemented a modified version of the Visible Burrow System (VBS), a semi-natural habitat providing burrows and an open area for mixed-sex rodent colonies, developed by Blanchard group (D. C. Blanchard et al., 2012; D. C. Blanchard et al., 1995; R. J. Blanchard, Dulloog, et al., 2001).

Moreover, considering the important role of genetic factors in the development of mental diseases, genetic manipulation in rodents can help to obtain suitable models resembling human neuropsychiatric symptoms. In this context, cadherin superfamily, originally characterized as
calcium-dependent cell-adhesion molecules, is now known to be involved in many biological processes, including cell recognition, cell signaling during embryogenesis and formation of neural circuits (Morishita & Yagi, 2007). In particular, protocadherin family, the largest subgroup within the cadherin superfamily, are predominantly expressed in the nervous system. Interestingly, recent evidence suggested that Protocadherin 9 (Pcdh9) is involved in sensory processing deficits and impaired social interaction (Bruining et al., 2015) that may be relevant in a wide variety of neuropsychiatric disorders, such as schizophrenia and ASD pathogenesis (Hirabayashi & Yagi, 2014; Morishita & Yagi, 2007). Moreover, Xiao and colleagues reported that the gene encoding Pcdh9 might be considered as a novel risk factor for Major Depressive Disorder (MDD) (Xiao, Zheng, et al., 2017).

In view of the sensory processing deficits that have been observed in Pcdh9 deficient mice, glutamate is the excitatory neurotransmitter of the thalamocortical inputs to the primary visual, auditory and somatosensory cortices, thus it is crucial for the cortical hierarchic structure controlling the interactions with the environment (Tecchio et al., 2011). Indeed, thalamocortical relay neurons receive ascending and descending glutamatergic excitatory inputs and are subjected to GABAergic inhibitory input which shapes the sensory information conveyed to the cortex (Vahle-Hinz, Detsch, Siemers, & Kochs, 2007).

In this study we investigated for the first time mixed-genotype colonies in VBS. In particular, we evaluated sociability and social withdrawal features in Pcdh9 Wild Type (WT), KO heterozygous (HET) and KO homozygous (HOM) mice, housed together in VBS. We also investigated GABAergic and glutamatergic alterations in the somatosensory cortex of Pcdh9 WT, KO HET and KO HOM mice.

5.2 Materials and Methods

Animals

Adult Pcdh9 WT, KO HET and KO HOM male and female mice aged 14-22 weeks were used in this study. Animals were bred in the animal facilities of the University of Groningen. Animals were housed in standard polypropylene cages, 34 cm x 18 cm x 14 cm, in a group of two mice in a temperature-controlled room (temperature 21 ± 2 °C). All subjects were maintained on a 12-h light/dark cycle, with access to water and standard chow ad libitum in their home cages. All
procedures were conducted in accordance with protocols approved by the University of Groningen.

**Generation and Breeding of Pcdh9-Knockout Mice**

Pcdh9-deficient mice were generated using a standard procedure (Bruining et al., 2015). Briefly, a targeting vector was designated to delete the second exon of the mouse Pcdh9 gene, which encodes extracellular, transmembrane, and part of cytoplasmic domains. The targeting vector was constructed by using RPCI-23BAC library (Genetycs, Tsukuba). The 17.2-kb genomic DNA fragment was cloned into pBRSDT. The 5’ homology arm was a HindIII/Sacl-digested 8.7-kb fragment, and a floxed PGK-neo positive selection marker was placed at its downstream. The 3’ homology arm was a Sacl/Nhel-digested 8.8-kb fragment, and a diphtheria toxin A fragment containing a poly-A signal was added as a negative selection marker. We obtained homologous recombinants using TT-2 embryonic stem cells. Mice possessing the neo cassette were produced using standard procedures for chimeric mouse production. Successful gene targeting was confirmed by Southern blot analysis. The isogenic mice were then used to generate time-mated HET breeding pairs to obtain WT and mutant mice from at least three different litters to mimic the background of the parental CS514 and C57BL/6J controls. Chimeric mice were crossed to C57BL/6J females. Initially, F1 hybrids from HET matings were generated. Repeated backcrossing with C57BL/6J mice (>10 generations) was conducted to ensure an isogenic C57BL/6J background (Bruining et al., 2015). For the Pcdh9 KO and WT mice all analyses were performed blind for Pcdh9 genotype.

**Genotyping**

Total RNA was isolated from mouse ear clips according to the manufacturer’s instructions using the TRizol procedure (BoomLab, Meppel, The Netherlands). RNA concentrations and purity were measured with a NanoDrop® spectrophotometer (Thermo Scientific, Eindhoven, The Netherlands). For conversion into cDNA, the cDNA Synthesis Kit (BoomLab, Meppel, The Netherlands) was used and the reverse transcription reaction was performed in a thermocycler (PTC-200, MJ Research) with a 3-step program: 10 min at 25°C followed by 60 min at 42°C and a final 5 min step at 85°C. The machine was set to cool down automatically to 4°C after the end of the final step. cDNA samples were then used immediately for Real-Time PCR. Real-Time PCR was performed by using DreamTaq polymerase in accordance with the manufacturer’s instructions (Thermo Scientific, Eindhoven, The Netherlands). For the target genes the primers used were Pcdh9R1 (5’- ACCAGTCTGAGACAAGGCT -3’), Pcdh9R22 (5’- TACCCGGTAGAATTGACCTGCAG -3’), Pcdh9F1 (5’-
GTGGCTGTCTCCACATAAGA -3'). The mixture was prepared by adding the cDNA sample, followed by dNTP mix (Thermo Scientific, Eindhoven, The Netherlands) and 10X primers for the target genes. The samples were then placed in thermal cycler machine and run following thermal cycling conditions: step 1: 94°C for 2 min, step 2: 94°C for 30 sec, step 3: 60°C for 45 sec, step 4: 72°C for 1 min, step 5: from step 2, 32x times, step 6: 72°C for 7 min, step 7: 10°C until the end. At the end of the reaction, the PCR mixture was loaded with 6x loading dye and run together with a DNA Marker on 1.5% agarose gel for electrophoresis (approx. 30 minutes at 90V). The following profiles can be expected: 350bp: Pcdh9 WT; 350bp and 500bp: Pcdh9 KO HET; 500bp: Pcdh9 KO HOM.

Apparatus
The VBS’ were built in-house at the University of Groningen, based on the design by Blanchard et al. (D. C. Blanchard et al., 1995). Extra chambers (nests) were added to better study the social dynamics. The system consisted of two parts: an open arena (50cm x 50cm) with two stations where animals had access to food and water ad libitum, and a burrow (50cm x 25cm) with 4 chambers and a corridor. The open arena was subjected to a 12:12 L/D cycle (ZT0 at 08:00, see figure 3) and was open to the outside. The burrow of the VBS was closed using a polycarbonate lid that functioned as an infrared-pass filter. Thus the burrow was in complete darkness at all time, resembling the natural environment. Within the burrow 2 big chambers (7,5cm x 12,5cm) and 2 small chambers (7,5cm x 7,5cm) were placed with a tunnel connecting them to each other and to the open arena (see Figure 1). Behaviour in the VBS was recorded using a Bassler Cam GigE monochrome infrared sensitive camera (acA1300-60gm). Thus, due to its infrared sensitivity, the camera not only recorded behaviour in the open arena, but also could capture behaviour in the burrow through the polycarbonate lid.
Experimental procedures

Animals were placed in the experimental room two weeks before the start of the experiments. Each colony consisted of 6 male mice, 2 Pcdh9 WT, 2 Pcdh9 KO HOM and 2 Pcdh9 KO HET, and 2 Pcdh9 WT female mice. Every colony contained no more than 2 littermates. Females were used to mimic the natural group-housed conditions in rodents. Females were previous sterilized by ligating the oviducts and leaving the ovaries intact in order to maintain the estrous cycle. Estrous cycle was monitored every day before the start of the experiments. Two days before the start of the experiment males were marked with a commercial crème-based hair dye (Garnier Olia B++ Super blonde) to facilitate individual recognition of the animals. Animals were housed in the system for 8 days. During the experiment, the animals were recorded continuously. The animals were weighted at the beginning and at the end of the experiment in order to leave them undisturbed in the system. We performed and analyzed a total of 3 mixed-genotypes colonies.

Behavioural ethogram

Social and non-social behaviours scored are described in Table 1.
Table 1

Behavioural ethogram describing all the different behaviour scored.

<table>
<thead>
<tr>
<th>BEHAVIOURS</th>
<th>DESCRIPTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Social Exploration</td>
<td>Sniffing another animal, following another animal, playing with another animal</td>
</tr>
<tr>
<td>Approach</td>
<td>Moving towards another animal</td>
</tr>
<tr>
<td>Aggression</td>
<td>When the subject is biting, chasing, fighting other animals</td>
</tr>
<tr>
<td>Avoidance</td>
<td>submissive and avoidance behaviour. Submissive reactions to aggressive behaviour (i.e. not fighting back/surrendering). Also moving/running away from aggressive encounters and social contact/approaches</td>
</tr>
<tr>
<td>Huddling</td>
<td>Resting/huddling while in contact with conspecifics. When the subject resumes activity for more than 5 seconds, behaviour is not considered part of resting/huddling</td>
</tr>
<tr>
<td>Sexual activity</td>
<td>Mounting Female</td>
</tr>
<tr>
<td>Passive/Receiving social contact</td>
<td>Receiving social contact is scored when an animal does not react to social exploration of another animal (i.e. passive social interaction)</td>
</tr>
<tr>
<td>Allogrooming</td>
<td>When an animal is grooming another animal</td>
</tr>
<tr>
<td>Autogrooming</td>
<td>When an animal is grooming itself</td>
</tr>
<tr>
<td>Feeding/Drinking</td>
<td>Feeding/drinking from the feeding station</td>
</tr>
<tr>
<td>Environmental Exploration</td>
<td>Animal explores the surrounding environment, behaviour is not aimed towards another animal. (e.g. digging, locomotion, sniffing the walls)</td>
</tr>
<tr>
<td>Alone Inactivity</td>
<td>Resting whilst not being in bodily contact with another animal. When the subject resumes activity for more than 5 seconds, behaviour is not considered part of resting</td>
</tr>
</tbody>
</table>

Behavioural analyses

Behaviour was analyzed using The Observed 13 XT (Noldus Information Technology, Wageningen, The Netherlands). Each colony was observed for 10 minutes of six hours divided over the day. The six hours were previously tested and then selected for their representation of the full day and to cover most of the activity phase of the animals. The first 10 minutes of these hours were tested for their accuracy in representing the full hour. A total of 3 mixed-genotype Pcdh9 colonies were scored manually for 5 days. Frequency and duration in seconds of every behaviour were scored for every 10 minutes of each of the chosen six hours and data were showed as frequency per day and
time spent per day. The data of the 5 days scored were summed and showed in the overall
behaviour in order to analyze strain differences.

**Post mortem analyses**
After 8 days of VBS colony housing, mice were immediately euthanized by cervical dislocation and
brains were collected, frozen in isopentane and stored at -80°C.
For the standard-housed measurements, 4 adult *Pcdh9* KO HET, KO HOM and WT male mice,
housed in standard cages, two per cage, were euthanized, brains were collected, frozen in
isopentane and stored at -80°C.

**HPLC quantifications**
GABA and glutamate concentrations in somatosensory cortex were determined by HPLC using
ODS-3 column (150 × 4.6 mm, 3 μm; INERTSIL) with fluorescence detection after derivatization
with ophthalaldehyde/mercaptopropionic acid (emission length, 4.60 nm; excitation length, 3.40
nm). The mobile phase gradient consisted of 50 mM sodium acetate buffer, pH 6.95, with
methanol increasing linearly from 2 to 30% (v/v) over 40 min. The flow rate was maintained by a
pump (JASCO, Tokyo, Japan) at 0.5 ml/min. Results were analyzed by Borwin software (version
1.50; Jasco) and substrate concentration was expressed as µM.

**Statistical analyses**
Frequency and duration of each behaviour were tested for normality and then analyzed per day
using Two-way ANOVA for repeated measures followed by a Bonferroni’s post-hoc test.
Differences in the overall behaviour and neurochemical data were tested for normality and then
analyzed using One-way ANOVA followed by Bonferroni’s post-hoc test. Results were expressed as
mean ± S.E.M. Statistical analyses were performed using Graph Pad 5.0 (GraphPad Software, San
Diego, CA) for Windows. Differences were considered statistically significant when P value was less
than 0.05.

**5.3 Results**
In order to evaluate *Pcdh9* genotype differences, we analyzed mixed-sex colonies formed by 2
*Pcdh9* WT, 2 *Pcdh9* KO HET and 2 *Pcdh9* KO HOM males, with 2 *Pcdh9* WT females, housed
together in VBS. Moreover, we measured GABA and glutamate content in the somatosensory
cortex of mice housed in the VBS colonies and mice housed in standard cages, for the three
different genotypes, respectively.
Social behaviours in mixed-genotype VBS colonies

We scored social exploration, huddling, aggression and sexual activity as social behaviours, for both frequency and duration. In particular, we found that duration and frequency of social exploration during the daily scoring and the overall were not significantly different among the three groups (Fig. 2A, Two-way ANOVA RM followed by Bonferroni, n.s.; Fig. 2B One-way ANOVA followed by Bonferroni, n.s.; Fig. 2C, Two-way ANOVA RM followed by Bonferroni, n.s.; Fig. 2D One-way ANOVA followed by Bonferroni, n.s.). In addition, our results did not show any difference in terms of huddling behaviour among the different genotypes, for both frequency and duration (Fig. 2E, Two-way ANOVA RM followed by Bonferroni, n.s.; Fig. 2F One-way ANOVA followed by Bonferroni, n.s.; Fig. 2G, Two-way ANOVA RM followed by Bonferroni, n.s.; Fig. 2H One-way ANOVA followed by Bonferroni, n.s.). Moreover, there were no differences in time spent performing aggression, and also in frequency of aggression, among Pcdh9 KO HOM, KO HET and WT (Fig. 2I, Two-way ANOVA RM followed by Bonferroni, n.s.; Fig. 2J One-way ANOVA followed by Bonferroni, n.s.; Fig. 2K, Two-way ANOVA RM followed by Bonferroni, n.s.; Fig. 2L One-way ANOVA followed by Bonferroni, n.s.). Ultimately, we did not find significant differences in time spent and frequency of sexual activity (Fig. 2M, Two-way ANOVA RM followed by Bonferroni, n.s.; Fig. 2N One-way ANOVA followed by Bonferroni, n.s.; Fig. 2O, Two-way ANOVA RM followed by Bonferroni, n.s.; Fig. 2P One-way ANOVA followed by Bonferroni, n.s.).
Figure 2 Duration and frequency of social behaviours in mixed-genotype Pcdh9 colonies. Time spent performing social exploration per day (A) in Pcdh9 KO HET (red line), KO HOM (blue line) and Pcdh9 WT (black line) mice. Two-way ANOVA RM followed by Bonferroni, n.s. Overall time spent performing social exploration (B) in Pcdh9 KO HOM (white bar), KO HET (gray bar) and WT (black bar) mice. One-Way ANOVA followed by Bonferroni, n.s. Frequency of social exploration per day (C) in Pcdh9 KO HET (red line), KO HOM (blue line) and Pcdh9 WT (black line) mice. Two-way ANOVA RM followed by Bonferroni, n.s. Overall frequency of social exploration (D) in Pcdh9 KO HOM (white bar), KO HET (gray bar) and WT (black bar) mice. One-Way ANOVA followed by Bonferroni, n.s. Time spent performing huddling per day (E) in Pcdh9 KO HET (red line), KO HOM (blue line) and Pcdh9 WT (black line) mice. Two-way ANOVA RM followed by Bonferroni, n.s. Overall time spent performing huddling (F) in Pcdh9 KO HOM (white bar), KO HET (gray bar) and WT (black bar) mice. One-Way ANOVA followed by Bonferroni, n.s. Frequency of huddling per day (G) in Pcdh9 KO HET (red line), KO HOM (blue line) and Pcdh9 WT (black line) mice. Two-way ANOVA RM followed by Bonferroni, n.s. Overall frequency of huddling (H) in Pcdh9 KO HOM (white bar), KO HET (gray bar) and WT (black bar) mice. One-Way ANOVA followed by Bonferroni, n.s. Time spent performing aggression per day (I) in Pcdh9 KO HET (red line), KO HOM (blue line) and Pcdh9 WT (black line) mice. Two-way ANOVA RM followed by Bonferroni, n.s.
Overall time spent performing aggression (J) in Pcdh9 KO HOM (white bar), KO HET (gray bar) and WT (black bar) mice. One-Way ANOVA followed by Bonferroni, n.s. Frequency of aggression per day (K) in Pcdh9 KO HET (red line), KO HOM (blue line) and Pcdh9 WT (black line) mice. Two-way ANOVA RM followed by Bonferroni, n.s. Overall frequency of aggression (L) in Pcdh9 KO HOM (white bar), KO HET (gray bar) and WT (black bar) mice. One-Way ANOVA followed by Bonferroni, n.s. Time spent performing sexual activity per day (M) in Pcdh9 KO HET (red line), KO HOM (blue line) and Pcdh9 WT (black line) mice. Two-way ANOVA RM followed by Bonferroni, n.s. Overall time spent performing sexual activity (N) in Pcdh9 KO HOM (white bar), KO HET (gray bar) and WT (black bar) mice. One-Way ANOVA followed by Bonferroni, n.s. Frequency of sexual activity per day (O) in Pcdh9 KO HET (red line), KO HOM (blue line) and Pcdh9 WT (black line) mice. Two-way ANOVA RM followed by Bonferroni, n.s. Overall frequency of sexual activity (P) in Pcdh9 KO HOM (white bar), KO HET (gray bar) and WT (black bar) mice. One-Way ANOVA followed by Bonferroni, n.s. Data are expressed as mean ± SEM (n=6 per group).

**Non-social behaviours in mixed-genotype VBS colonies**

We scored environmental exploration, alone inactivity, avoidance and passive/receiving social contact as non-social behaviours, for both frequency and duration. In particular, we found that duration and frequency of environmental exploration during the daily scoring and the overall were not significantly different among the three groups (Fig. 3A, Two-way ANOVA RM followed by Bonferroni, n.s.; Fig. 3B One-way ANOVA followed by Bonferroni, n.s.; Fig. 3C, Two-way ANOVA RM followed by Bonferroni, n.s.; Fig. 3D One-way ANOVA followed by Bonferroni, n.s.). In addition, our results did not show any difference in terms of alone inactivity among the different genotypes, for both frequency and duration (Fig. 3E, Two-way ANOVA RM followed by Bonferroni, n.s.; Fig. 3F One-way ANOVA followed by Bonferroni, n.s.; Fig. 3G, Two-way ANOVA RM followed by Bonferroni, n.s.; Fig. 3H One-way ANOVA followed by Bonferroni, n.s.). Moreover, there were no differences in time spent performing avoidance behaviour, and also in frequency of avoidance, among Pcdh9 KO HOM, KO HET and WT (Fig. 3I, Two-way ANOVA RM followed by Bonferroni, n.s.; Fig. 3J One-way ANOVA followed by Bonferroni, n.s.; Fig. 3K, Two-way ANOVA RM followed by Bonferroni, n.s.; Fig. 3L One-way ANOVA followed by Bonferroni, n.s.). Ultimately, we did not find significant differences in time spent and frequency of passive/receiving social contact behaviour (Fig. 3M, Two-way ANOVA RM followed by Bonferroni, n.s.; Fig. 3N One-way ANOVA followed by Bonferroni, n.s.; Fig. 3O, Two-way ANOVA RM followed by Bonferroni, n.s.; Fig. 3P One-way ANOVA followed by Bonferroni, n.s.).
Figure 3 Duration and frequency of non-social behaviours in mixed-genotype Pcdh9 colonies. Time spent performing environmental exploration per day (A) in Pcdh9 KO HET (red line), KO HOM (blue line) and Pcdh9 WT (black line) mice. Two-way ANOVA RM followed by Bonferroni, n.s. Overall time spent performing environmental exploration (B) in Pcdh9 KO HOM (white bar), KO HET (gray bar) and WT (black bar) mice. One-Way ANOVA followed by Bonferroni, n.s. Frequency of environmental exploration per day (C) in Pcdh9 KO HET (red line), KO HOM (blue line) and Pcdh9 WT (black line) mice. Two-way ANOVA RM followed by Bonferroni, n.s. Overall frequency of environmental exploration (D) in Pcdh9 KO HOM (white bar), KO HET (gray bar) and WT (black bar) mice. One-Way ANOVA followed by Bonferroni, n.s. Time spent performing alone inactivity per day (E) in Pcdh9 KO HET (red line), KO HOM (blue line) and Pcdh9 WT (black line) mice. Two-way ANOVA RM followed by Bonferroni, n.s. Overall time spent performing alone inactivity (F) in Pcdh9 KO HOM (white bar), KO HET (gray bar) and WT (black bar) mice. One-Way ANOVA followed by Bonferroni, n.s. Frequency of alone inactivity per day (G) in Pcdh9 KO HET (red line), KO HOM (blue line) and Pcdh9 WT (black line) mice. Two-way ANOVA RM followed by Bonferroni, n.s. Overall frequency of alone inactivity (H) in Pcdh9 KO HOM (white bar), KO HET (gray bar) and WT (black bar) mice. One-Way ANOVA followed by Bonferroni, n.s. Time spent performing avoidance per day (I) in Pcdh9 KO HET (red line), KO HOM (blue line) and Pcdh9 WT (black line) mice. Two-way ANOVA RM followed by Bonferroni, n.s. Overall time spent performing avoidance (J) in Pcdh9 KO HOM (white bar), KO HET (gray bar) and WT (black bar) mice. One-Way ANOVA followed by Bonferroni, n.s. Frequency of
avoidance per day (K) in Pcdh9 KO HET (red line), KO HOM (blue line) and Pcdh9 WT (black line) mice. Two-way ANOVA RM followed by Bonferroni, n.s. Overall frequency of avoidance (L) in Pcdh9 KO HOM (white bar), KO HET (gray bar) and WT (black bar) mice. One-Way ANOVA followed by Bonferroni, n.s. Time spent performing passive/receiving social contact per day (M) in Pcdh9 KO HET (red line), KO HOM (blue line) and Pcdh9 WT (black line) mice. Two-way ANOVA RM followed by Bonferroni, n.s. Overall time spent performing passive/receiving social contact (N) in Pcdh9 KO HOM (white bar), KO HET (gray bar) and WT (black bar) mice. One-Way ANOVA followed by Bonferroni, n.s. Frequency of passive/receiving social contact per day (O) in Pcdh9 KO HET (red line), KO HOM (blue line) and Pcdh9 WT (black line) mice. Two-way ANOVA RM followed by Bonferroni, n.s. Overall frequency of passive/receiving social contact (P) in Pcdh9 KO HOM (white bar), KO HET (gray bar) and WT (black bar) mice. One-Way ANOVA followed by Bonferroni, n.s. Data are expressed as mean ± SEM (n=6 per group).

**Increased glutamate content in somatosensory cortex in standard-housing conditions, but not in VBS colony housing**

We measured GABA and glutamate content in the somatosensory cortex of mixed-genotype VBS colonies and Pcdh9 WT, KO HET and KO HOM mice housed in standard cages, in order to investigate genotype differences.

Our results showed that no genotype differences were detected in GABA content in standard-housed mice (Fig. 4A, One-way ANOVA followed by Bonferroni, n.s.), while there was a significant increase in glutamate levels in somatosensory cortex of Pcdh9 KO HOM compare to KO HET and WT mice (Fig. 4B, One-way ANOVA followed by Bonferroni, F=15.72, P<0.01 KO HOM vs. KO HET and WT). Moreover, we did not found any difference in GABA and glutamate contents in somatosensory cortex among the three different groups (Fig. 4C-D, One-way ANOVA followed by Bonferroni, n.s.).
Figure 4 Effect of VBS colony housing on GABA and glutamate levels in somatosensory cortex of Pcdh9 KO HOM, HET and WT mice. GABA (A) levels in somatosensory cortex of Pcdh9 WT (white bar), KO HET (gray bar) and KO HOM (black bar) mice housed in standard cages. One-Way ANOVA followed by Bonferroni, n.s. Glutamate (B) levels in somatosensory cortex of Pcdh9 WT (white bar), KO HET (gray bar) and KO HOM (black bar) mice housed in standard cages. One-Way ANOVA followed by Bonferroni, **p<0.01 vs. WT, ##p<0.01 vs. KO HET. GABA (C) and glutamate (D) levels in somatosensory cortex of Pcdh9 WT (white bar), KO HET (gray bar) and KO HOM (black bar) mice housed in VBS colonies. One-Way ANOVA followed by Bonferroni, n.s.

5.4 Discussion

In the present study, we investigated social dynamics in mixed-genotype and mixed-sex Pcdh9 colonies in the VBS paradigm. Our preliminary analysis showed that there were no differences in terms of social behaviours and non-social behaviours among the three genotypes, for both duration and frequency. Additional analyses (e.g., including the implementation of location data and increasing the number of colonies tested) are needed to further strengthen these data.
Moreover, no genotype differences were detected in GABA content in somatosensory cortex, for both standard housing condition and VBS colony housing. Interestingly, glutamate levels were significantly increased in *Pcdh9* KO HOM mice housed in standard cages, while this increase was not found in HOM *Pcdh9*-deficient mice housed in the VBS.

We investigated social and non-social behaviours in mixed-sex and mixed-genotype colonies, using a modified VBS version, with four nests in a continuously dark burrow and a large open arena, mimicking the natural rodent housing condition. In addition, we housed together *Pcdh9* WT and KO HET and HOM animals, aiming to analyze genotype differences.

In particular, our results did not show genotype differences in social exploration, huddling, aggression and sexual activity. Thus, there were no differences in terms of social behaviours duration and frequency. Moreover, also non-social behaviours, in particular environmental exploration, alone inactivity, avoidance and passive/receiving social contact, did not show any significant genotype difference.

Interestingly, *Pcdh9* has been recently identified as an interesting candidate gene associated with ASD (Hirabayashi & Yagi, 2014; Morishita & Yagi, 2007). In this regard, Bruining and colleagues demonstrated that HOM *Pcdh9*-deficient mice show specific deficits in long-term social recognition, accompanied by additional impairments in sensorimotor development reflected by early touch-evoked biting, rotarod performance, and sensory gating deficits (Bruining et al., 2015). However, the recognition deficits in *Pcdh9* KO mice were not associated with alterations in perception, multi-trial discrimination learning, sociability, behavioral flexibility or fear memory (Bruining et al., 2015). In line with our results, also Bruining and colleagues did not find genotype differences in three-chamber test, the most used dyadic test to assess sociability in rodents.

However, we did not investigated sociability in a standard environment or social behaviours among *Pcdh9* colonies composed by the same genotype, thus future studies are required to better understand HOM *Pcdh9* KO social phenotype without the presence of social stimuli. Indeed, VBS colonies formed by mixed-genotype and mixed-sex mice are considered highly social environment, hence we hypothesized that strong social stimuli might be helpful to improve probable social deficits.

Social deficits are an important core symptoms of ASD and schizophrenia. The positive effect of the social stimuli in the mixed-genotype VBS colonies might reflect the importance of the behavioural therapy in patients. In accordance with our hypothesis, occupational therapists,
together with teachers, support autistic student participation in classrooms in order to stimulate sociability and increase socialization (Mills & Chapparo, 2017). Moreover, a recent study reported that trained parents, working together with their children, are able to improve their social skills (Dogan et al., 2017), suggesting the beneficial effects that social stimuli can have. Furthermore, social deficits, in particular social withdrawal and anhedonia, are considered negative symptoms of schizophrenia. These symptoms, associated with cognitive (such as attention and memory deficits) and positive symptoms (such as delusions and hallucinations) significantly impair patients’ personal and professional lives (Balhara & Verma, 2012). In particular, negative symptoms often lead to homelessness and are thought to be the primary driver for suicide in schizophrenics (Balhara & Verma, 2012).

In addition, a number of clinical evidence support the importance of employment among schizophrenic patients (Carmona, Gomez-Benito, Huedo-Medina, & Rojo, 2017; Llerena, Reddy, & Kern, 2017; Martini et al., 2017). In particular, work is considered one of the main forms of social organization and few individuals with mental illnesses find work opportunities (Martini et al., 2017). In an interesting clinical study, it has been reported that negative symptoms hinder job attainment and work outcomes in people with schizophrenia, rather than cognitive and positive symptoms, thus an enhanced understanding of the domains of negative symptoms is vital in order to develop treatments that translate into better employment outcomes (Llerena et al., 2017). Antipsychotic pharmacotherapy has shown some success in alleviating positive symptoms, although treatment options for negative and cognitive symptoms remain very limited. Consequently, there is an urgent need to decipher the underlying causes of these symptoms and develop new pharmacological strategies.

In this regard, we have previously shown the suitability of VBS to investigate social withdrawal features and their underlying neurobiology in a mice model of ASD (Bove et al., submitted). Thus, we quantified GABA and glutamate content in somatosensory cortex of Pcdh9 WT, HOM KO and HET KO housed in VBS colonies and in standard cages. Our results showed that there were no differences in GABA content among the three genotypes in both VBS colonies and standard cages. Otherwise, glutamate was significantly increased only in HOM Pcdh9-deficient mice housed in standard cages, while no genotype differences were found in glutamate levels among VBS colonies. In this regard, it has been reported that altered glutamate transmission is a common feature of many neuropsychiatric conditions, including schizophrenia (O'Donovan,
Sullivan, & McCullumsmith, 2017). Moreover, a number of neuropsychiatric diseases associated to schizophrenia-like symptoms are characterized by alterations in sensory processing and perception (Gonzalez-Maeso et al., 2008). In addition, recent evidence reported that drugs interacting with metabotropic glutamate receptors show potential for the treatment of neuropsychiatric diseases (Aghajanian & Marek, 2000; Marek, 2004; Patil et al., 2007). Thus, glutamate neurotransmission in somatosensory cortex area might participate to the development of schizophrenia-like symptoms. Interestingly, a recent evidence reported that a plant-derived compound was able to reduce glutamate-evoked excitotoxicity in the somatosensory cortex acting through the inhibition of non-NMDA glutamate receptors (Borbely et al., 2016). Thus, attenuation of glutamatergic tone might represent a new treatment strategy in different brain diseases, such as schizophrenia, autism, bipolar disorders, major depression and mental retardation (Contractor, Mulle, & Swanson, 2011).

Intriguingly, the VBS, beyond its employment as a tool to assess sociability in a translational way, might also be used as a behavioural paradigm to further test pharmacological treatments aiming at restoring social dysfunctions commonly occurring in several neuropsychiatric disorders, such as social withdrawal.

To the best of our knowledge, this is the first study that evaluated mixed-genotype colonies in VBS. In particular, we showed no differences in sociability and social withdrawal features among Pcdh9 WT, KO HOM and KO HET, indicating no disrupted sociability of Pcdh9-deficient mice when housed together with WT in the VBS. Moreover, the glutamate increase found in HOM Pcdh9-deficient mice housed in standard cages was not retrieved in VBS colony housing, suggesting a beneficial effect of this highly social environment on glutamate increase induced by Pcdh9 genetic ablation.