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# **Autistic-like syndrome in mu opioid receptor null mice is relieved by facilitated mGluR4 activity**

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## **Abstract**

The etiology of Autism Spectrum Disorders (ASDs) remains largely unknown. Identifying vulnerability genes for autism represents a major challenge in the field, and allows the development of animal models for translational research. Mice lacking the mu opioid receptor gene (*Oprm1*<sup>-/-</sup>) were recently proposed as a monogenic mouse model of autism, based on severe deficits in social behavior and communication skills. We confirm this hypothesis by showing that adult *Oprm1*<sup>-/-</sup> animals recapitulate core and multiple comorbid behavioral symptoms of autism, and also display anatomical, neurochemical and genetic landmarks of the disease. Chronic facilitation of mGluR4 signaling, which we identified as a novel pharmacological target in ASDs in these mice, was more efficient in alleviating behavioral deficits than the reference molecule risperidone. Altogether, our data provide first evidence that disrupted mu opioid receptor signalling is sufficient to trigger a comprehensive autistic syndrome, maybe through blunted social reward processes, and this mouse model opens promising avenues for therapeutic innovation.

## **Introduction**

Autism Spectrum Disorders (ASDs) are complex neurodevelopmental diseases with high heterogeneity and heritability, whose diagnosis is based on two types of behavioral symptoms: impaired social reciprocity and communication, and restricted range of behaviors, interests and activities (DSM-5). Secondary - comorbid - symptoms vary substantially, and include anxiety disorders, cognitive and motor deficits, aggressive behavior and epileptic episodes (Johnson and Myers 2007; Mazurek et al. 2013; Pouw et al. 2013; Robinson 2012; Veenstra-VanderWeele and Blakely 2012; White et al. 2012; Whyatt and Craig 2013). Heterogeneity in ASDs points toward multiple primary causes, with genetic factors playing a critical role. Large scale genetic studies in patients and preclinical work using animal models

have ~~indeed~~ identified over 280 candidate vulnerability genes for ASD (SFARIgene<sup>2.0</sup> database, <https://gene.sfari.org/autdb/Welcome.do>) (Buxbaum et al. 2012; Delorme et al. 2013; Ecker et al. 2012a; State and Levitt 2011), and suggest that altered synaptic function and disrupted excitation-inhibition balance contribute to the pathology. Neuroimaging studies have confirmed atypical brain connectivity in ASD patients (Ecker et al. 2012b; Just et al. 2012). The precise cause of these troubles, however, remains largely unknown.

Given the high homology between mouse and human genomes and feasibility of genetic modifications in mice, interest in developing genetic mouse models of ASDs has raised recently, together with the development of dedicated behavioral assessment (Buxbaum et al. 2012; Crawley 2007; Robertson and Feng 2011). These models ought to demonstrate face (similarity with clinical symptoms), construct (similarity in causal mechanisms) and predictive (similarity in response to therapeutics) validity; none of the currently available ASD models, however, fully matches such criteria. Nevertheless, they have contributed to a better understanding of the neurobiological substrates of autism and to the identification of novel promising therapeutic trails.

Recently, mice lacking the mu opioid receptor gene (*Oprm1*<sup>-/-</sup>) were proposed as a monogenic model of autism (Oddi et al. 2013). These animals indeed demonstrate major deficits in social behavior: reduced maternal attachment in mouse pups (Moles et al. 2004), altered social reward in juvenile mice (Cinque et al. 2012) and blunted response to female vocalizations in adult males (Wohr et al. 2011). Thus, animal research highlights the critical role of mu opioid receptors in social responses, and pharmacological effects of mu opioid drugs in social play and adult attachment have been shown in rodents (Burkett et al. 2011; Trezza et al. 2011). In humans, neural sensitivity to social rejection (Way et al. 2009) and social hedonic capacity (Troisi et al. 2011) show significant association with a common variant (A118G) of the mu opioid receptor gene (*Oprm1*), suggesting a role of this receptor in

modulating social reward. Consistent with this, social acceptance activates mu opioid receptors in the ventral striatum, activation of which predicting the desire for social interaction (Hsu et al. 2013). Finally, genetic studies have detected discrete invalidating mutations of the *Oprm1* gene in ASD patients (SFARIgene<sup>2.0</sup> database), indicating that such invalidation may be sufficient to trigger an autistic syndrome, and providing construct validity for the *Oprm1*<sup>-/-</sup> mouse model. Whether the absence of mu opioid receptor signaling could lead to a comprehensive autistic-like phenotype in the adult animal, however, has not been tested yet. Here, we assessed behavioral, neurochemical, anatomical and genetic features of ASDs in these animals and, based on gene expression analysis, identified an entirely novel pharmacological target for therapeutic intervention in this pathology.

## **Material and Methods**

### **Subjects**

Male and female *Oprm1*<sup>+/+</sup> and *Oprm1*<sup>-/-</sup> mice (Matthes et al. 1996) aged 6-8-weeks were bred in-house on an identical hybrid background: 50% 129SVPas - 50% C57BL/6J. For the purpose of the present study, *Oprm1*<sup>+/+</sup> and *Oprm1*<sup>-/-</sup> pups were nurtured by *Oprm1*<sup>+/+</sup> and *Oprm1*<sup>-/-</sup> parents, respectively, except for cross-fostering experiment. Except otherwise stated, animals were group housed and maintained on a 12hr light/dark cycle (lights on at 7:00 AM) at controlled temperature (21±1°C); food and water were available *ad libitum*. Experiments were analyzed blind to genotypes. Experimental procedures were reviewed and approved by the Comité Régional d’Ethique en Matière d’Expérimentation Animale de Strasbourg (CREMEAS, 2003-10-08-[1]-58).

## **Behavioral experiments**

Detailed behavioral protocols are described in Supplementary Material and Methods. Social abilities were explored using the direct social interaction, the three-chamber and the nest building tests; aggressiveness was evaluated using the resident-intruder test. Stereotyped behavior was assessed by scoring motor stereotypies and measuring spontaneous alternation in a Y-maze. Anxiety-like behavior was evaluated in the marble burying and novelty-suppressed feeding tests. Motor function was assessed using the string and grip tests, skill motor learning task and foot print analysis. Sensitivity to seizures was assessed by scoring pentyhene tetrazole-induced convulsions. Experiments were performed on separate cohorts of naïve animals (Figures 1 and 2), except for tests exploring motor function (string test, grip test, accelerating rotarod, foot print analysis, in this order), cross-fostering experiment (Figure S3) and pharmacological experiments (Figures 4 and 5), where behavioral testing was performed in a battery (time lines in Figures S3a and S5a,b). Equivalent numbers of male and female animals were used in each experiment, except for the resident/intruder test, performed only in males.

## **Real-time quantitative PCR analysis**

Brains were removed and placed into a brain matrix (ASI Instruments, Warren, MI, USA). CPu, NAc, CeA and VTA were punched out from 1mm-thick slices. Prefrontal cortex (PFC) and MeA were dissected from two consecutive slices. Tissues were immediately frozen on dry ice and kept at -80°C until use. When assessing transcript levels of a collection of genes of interest, we pooled tissue from one male and one female mouse (n=5 samples/genotype) for each brain structure and each genotype, as no significant effect of gender was detected on mutant phenotype. When assessing transcription of immediate early genes *C-fos* and *Egr1* after social interaction, we processed each sample individually to parallel individual

behavioral data (n=7-8 samples/genotype and treatment). RNA was extracted and purified using the MIRNeasy mini-kit (Qiagen, Courtaboeuf, France). cDNA was synthesized using the first-strand Superscript II kit (Invitrogen®, Life Technologies, Saint Thomas, France). qRT-PCR was performed as previously described (Le Merrer et al. 2013). Primer sequences are displayed in Table S1.

## **Drugs**

Pentylenetetrazole (PTZ, Sigma-Aldrich, Saint-Quentin, France) was administered once a week at escalating doses (vehicle, 25, 37.5, 50 mg/kg i.p.). Scoring was performed immediately after treatment. Risperidone (Sigma-Aldrich, Saint-Quentin, France) at 0.2mg/kg i.p. (Penagarikano et al. 2011) was administered 60 min prior testing. VU0155041 (Tocris, Bristol, UK) at 5mg/kg i.p. (Duvoisin et al. 2011) was injected 120 min before testing. When risperidone and VU0155041 effects were compared to the same vehicle-treated group, control animals were tested 60 min after vehicle injection. All compounds were administered in a volume of 10ml/kg; vehicle was NaCl 0.9 %.

## **Statistical analyses**

Statistical analyses were performed using Statistica 9.0 software (StatSoft, Maisons-Alfort, France). For all comparisons, values of  $p < 0.05$  were considered as significant. Statistical significance in behavioral, immunohistochemical, electron microscopy and monoamine concentration experiments was assessed using two to four-way analysis of variance (genotype, treatment, trial and session effects) followed by Newman-Keules post-hoc test, except for nesting and convulsion scores, which significance was tested using the non-parametric Kruskal-Wallis' analysis of variance. A standard principal component analysis (PCA) (Le Merrer et al. 2012) was performed on behavioral and Fos count data. Loadings for

each extracted principal component (PC) are quoted in Table S3. We considered the two first extracted PCs (PC1 and PC2) for schematic representation. Significance of qRT-PCR results was assessed after transformation using a one-sample t-test, as previously described. Unsupervised clustering analysis was performed on the median of transformed qPCR data using complete linkage with correlation distance (Pearson correlation) for genes and brain region (Cluster 3.0 and Treeview software).

Detailed protocols for Fos immunohistochemistry, electron microscopy and biogenic amines dosage are described in Supplementary Material and Methods.

## Results

### ***Oprm1*<sup>-/-</sup> mice recapitulate core symptoms of autism**

Deficient social interactions are the most remarkable core symptom of autism in humans. We assessed social abilities in *Oprm1*<sup>-/-</sup> mice using four tests. In the direct social interaction test, *Oprm1*<sup>-/-</sup> mice spent less time in close interaction with a naive wild-type (WT) conspecific compared to *Oprm1*<sup>+/+</sup> controls, due to lower frequency and shorter duration of nose and paw contacts. Similarly, the frequency of followings was reduced in mutants. In contrast, these mice self-groomed more often, particularly after social contact, and for longer than controls, without other detectable motor stereotypies (Figures 1a and S1a, statistics in Table S2). In the three-chamber test, *Oprm1*<sup>+/+</sup> but not *Oprm1*<sup>-/-</sup> animals spent more time in nose contact with a conspecific versus a toy mouse during the interaction phase. When the toy mouse was replaced by a novel conspecific (recognition phase), *Oprm1*<sup>+/+</sup> but not *Oprm1*<sup>-/-</sup> mice spent more time interacting with this unfamiliar animal (Figures 1b and S1b, Table S2). Defective recognition of a novel social partner did not result from general alteration of recognition abilities, as object recognition was preserved in mutant animals (Figure S1c), or from deficient olfactory perception, as shown in previous studies (Cinque et al. 2012; Moles et al.

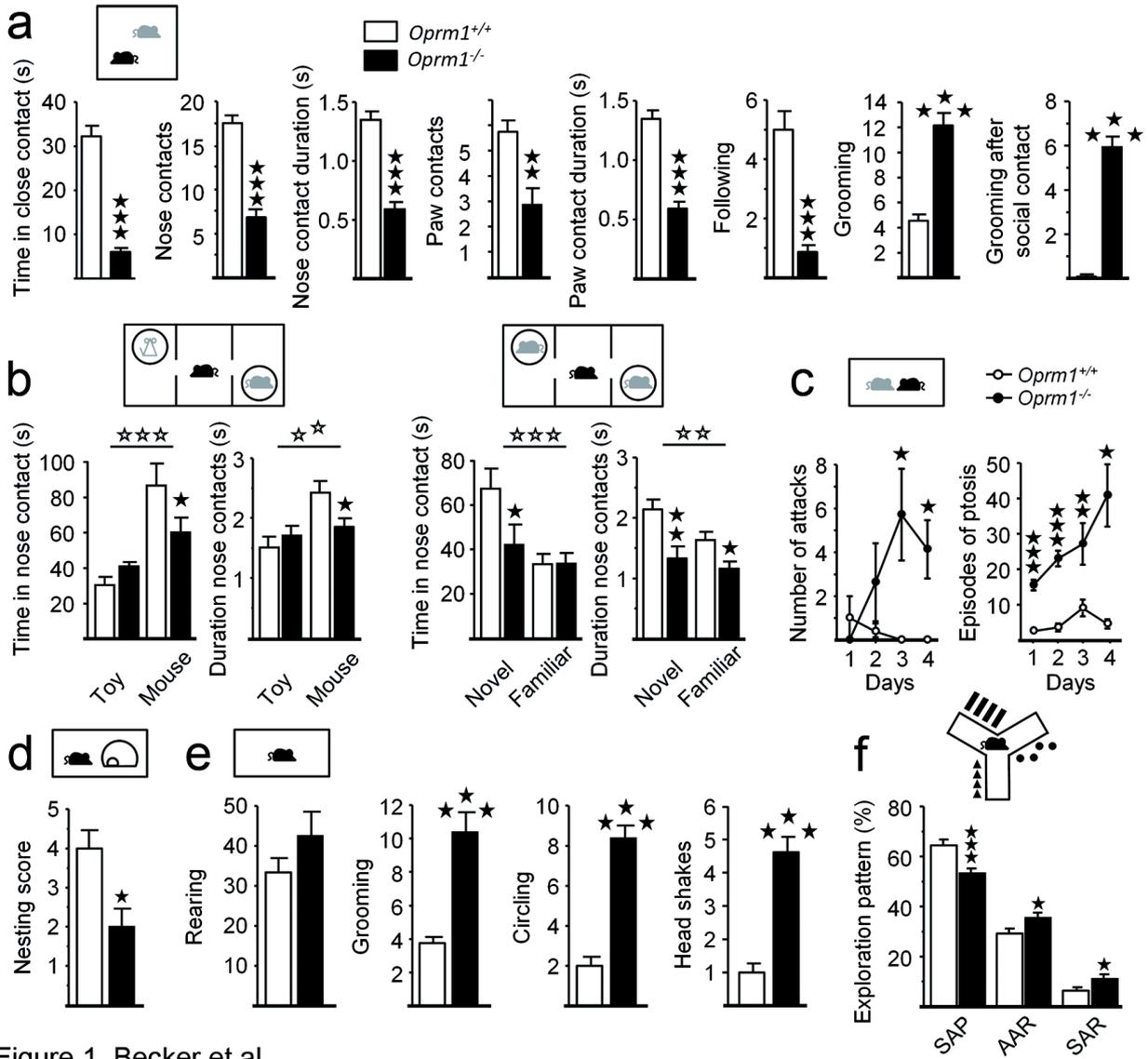


Figure 1. Becker et al.

**Figure 1. *Oprm1*<sup>-/-</sup> mice show core symptoms of autism.** (a) In the direct social interaction test, *Oprm1*<sup>-/-</sup> animals show decreased time in close social contact, due to lower frequency and duration of nose and paw contacts. Following was decreased whereas self-grooming, especially after social contact, was increased in mutants (n=8 per genotype). (b) In the three-chamber task, WT controls but not *Oprm1*<sup>-/-</sup> mice spend more time interacting with a mouse rather than a toy (interaction phase), or a novel rather than a familiar conspecific (recognition phase) (n=13-14 per genotype). (c) Male *Oprm1*<sup>-/-</sup> animals are more aggressive in the resident-intruder test, with increased numbers of attacks and episodes of ptosis (n=8 per genotype). (d) *Oprm1*<sup>-/-</sup> mice show impaired ability to build a nest (n=8 per genotype). (e) *Oprm1*<sup>-/-</sup> mice display motor stereotypies, with increased rearing, grooming, circling and head shakes (n=8 per genotype). (f) Mutant mice show deficient patterns of exploration in a Y-maze (n=16 per genotype). Spontaneous alternations (SPA) are decreased in *Oprm1*<sup>-/-</sup> animals; alternate arm returns (AAR) and same arm returns (SAR) are increased. Data are presented as mean ± SEM. Solid stars: genotype effect, open stars: stimulus effect. One star p<0.05, two stars p<0.01, three stars p<0.001. See also Figure S1.

2004). We observed that male *Oprm1*<sup>-/-</sup> mice displayed signs of aggressiveness in the three-chamber test. We thus assessed the presence of aggressive behavior in these animals using the resident-intruder test. Animals were isolated 4 weeks before a naïve male was introduced in their home cage, daily for 4 days. In contrast with *Oprm1*<sup>+/+</sup> controls, mutant animals engaged an increasing number of attacks towards the intruder mouse over sessions with shortening attack latency, together with increasing occurrence of ptosis episodes, a sign of aggressiveness in male mice (Defensor et al. 2012) (Figures 1c and S1d, Table S2). Moreover, mutant mice took significantly longer to leave their nest than controls during the first 3 sessions, indicative of exacerbated defensive aggressiveness (Rawleigh and Kemble 1992) (Figure S1d). Thus aggressive behavior, a symptom often associated with ASDs, is increased in *Oprm1*<sup>-/-</sup> animals. Finally, these mice revealed impaired ability to build a nest in the nesting behavior test (Figure 1d, Table S1). Together, these results indicate that adult *Oprm1*<sup>-/-</sup> mice display abnormal social abilities, prolonging a recent report of social behavior deficits in juvenile animals (Cinque et al. 2012).

Perseverative, stereotyped behaviors represent another major feature of ASDs, which we tested in mutant mice. When isolated in a novel standard cage, *Oprm1*<sup>-/-</sup> mice displayed more frequent grooming, circling and head shakes but not rearing or burying, than *Oprm1*<sup>+/+</sup> animals (Figures 1e and S1e), suggestive of stereotyped behaviors. We also assessed flexibility and perseveration by performing a continuous spontaneous alternation task in a Y-maze. Mutant animals showed decreased alternation, with increased frequency of alternate and same arm returns (Figure 1e). Increased stereotypic movements associated with reduced behavioral flexibility in *Oprm1*<sup>-/-</sup> mice therefore completed autistic-like core symptoms in mutant mice.

### ***Oprm1*<sup>-/-</sup> mice display multiple comorbid symptoms of autism**

The autistic syndrome also includes several comorbid symptoms with variable expression (reviewed in (Veenstra-VanderWeele and Blakely 2012)). We first evaluated anxiety levels, as anxiety disorders are commonly associated to ASD. Previous studies have evidenced lower anxiety levels in mutant animals in the elevated plus-maze test (Filliol et al. 2000; Ide et al. 2010). In the present work, we assessed anxiety-like behavior in additional paradigms, the marble burying test and the novelty-suppressed feeding test (NSF), which tax different aspects of anxiety. In the former, mutant animals buried more marbles than controls (Genotype:  $F_{1,16}=6.5$ ,  $p<0.05$ ; Gender:  $F_{1,16}<1$ , NS) (Figure 2a), demonstrating increased anxiety-induced stereotypies (Albelda and Joel 2012). In the NSF, a conflict test challenging approach/avoidance behavior (Aupperle and Paulus 2010), *Oprm1*<sup>-/-</sup> mice took longer to start feeding in the arena center (Genotype:  $F_{1,16}=49.9$ ,  $p<0.001$ ; Gender:  $F_{1,16}<1$ , NS), and ate less chow when returned to their home cage (Genotype:  $F_{1,16}=20.9$ ,  $p<0.001$ ; Gender:  $F_{1,16}<1$ , NS) compared to controls (Figure 2b). *Oprm1*<sup>-/-</sup> mice thus show increased conflict anxiety, which involves widespread neuronal activation (Aupperle and Paulus 2010). We measured such activation using Fos expression as a marker. When compared to *Oprm1*<sup>+/+</sup>, *Oprm1*<sup>-/-</sup> mice displayed higher Fos expression in brain regions involved in the control of anxiety: CeA, CA1, LS, BNST, Cg2 and DR. In contrast, Fos labeling was lower in regions that modulate reward and motivation processes: VTA and LH (Figure 2c,d; Table S2). To correlate behavioral and Fos responses, we performed a PCA on NSF data (Figure 2e, left panel: variables' space, right panel: subjects' space). Interestingly, Fos expression in CeA correlated tightly with the latency to eat, an anxiety parameter, whereas Fos expression in VTA clustered with food intake, a motivation parameter, opposed along a first principal component (PC1, Table S3). Remarkably, projection in the subjects' space (Figure 2e right) clearly dissociated *Oprm1*<sup>+/+</sup> from *Oprm1*<sup>-/-</sup> individual mice, demonstrating distinct patterns of behavioral responses and

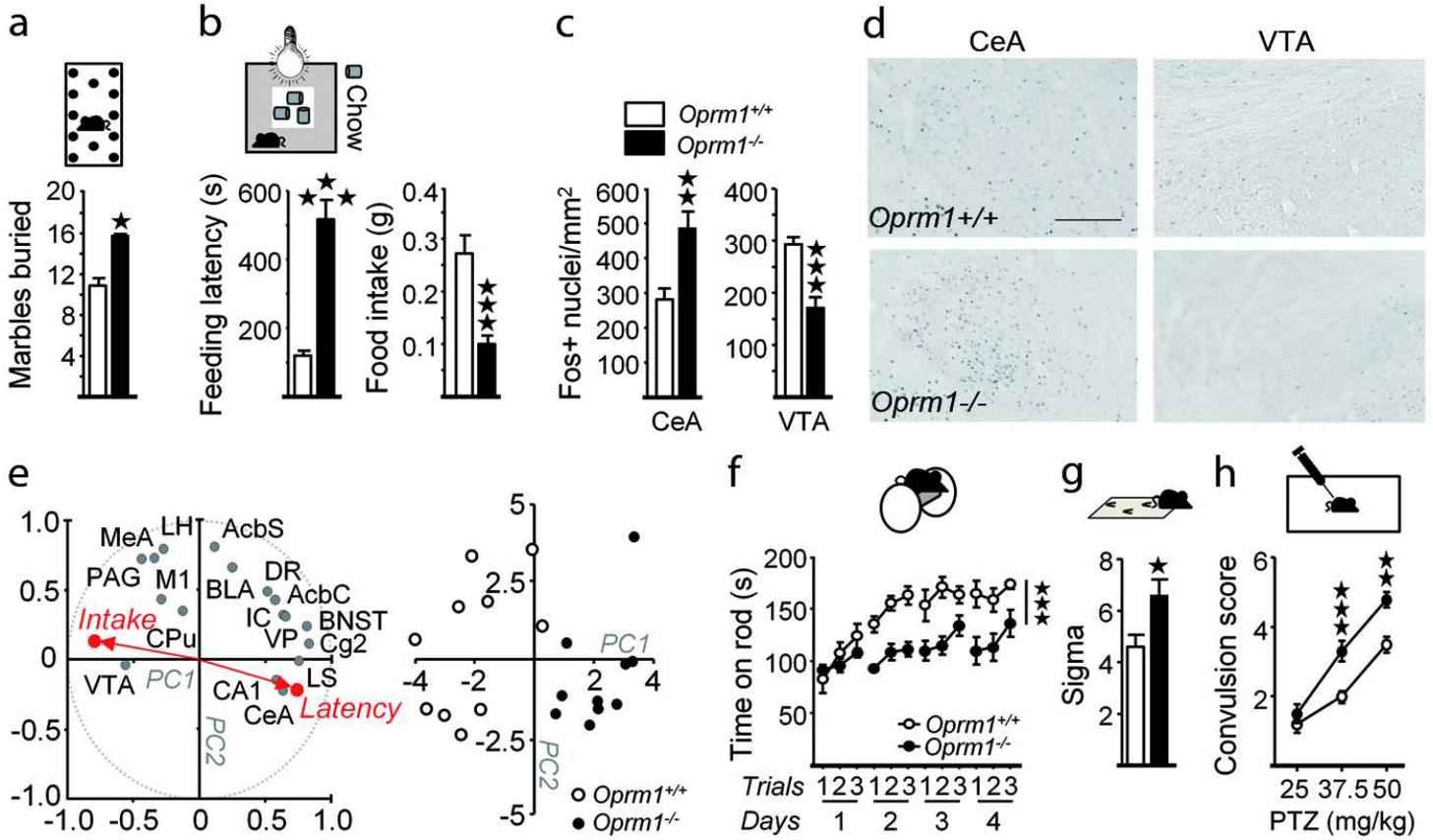


Figure 2. Becker et al.

**Figure 2. *Oprm1*<sup>-/-</sup> mice display multiple secondary symptoms of autism.**

(a) Defensive anxiety is higher in mutant animals, hiding more marbles than controls in the marble-burying test (n=10 per genotype). (b) Conflict anxiety also is increased, as shown by longer feeding latencies in the novelty-suppressed feeding (NSF) test (n=10 per genotype). (c) and (d) Increased anxiety of *Oprm1*<sup>-/-</sup> mice in NSF is associated with increased number of Fos positive (Fos+) nuclei in the central nucleus of the amygdala (CeA) and decreased Fos staining in the ventral tegmental area (VTA). (e) Principal component analysis on NSF data including behavioral (red dots) and Fos (grey dots) responses opposes food intake with latency to eat along the first principal component (PC1, 31.7% of variance). Fos expression in brain regions implicated in the regulation of anxiety-like behavior clusters with the latency parameter, and Fos-staining in neural circuits involved in the control of reward processes correlates with food intake (left panel: variables' space). *Oprm1*<sup>+/+</sup> and *Oprm1*<sup>-/-</sup> individual mice are dissociated in the subjects' space (right panel). (f) and (g) Motor function is altered in mutant animals. *Oprm1*<sup>-/-</sup> mice show impaired motor performance in the accelerating rotarod task (n=7 per genotype) and higher variability of pace size (sigma) in the footprint test (n=15-16 per genotype). (h) Sensitivity to pentylenetetrazole-induced seizures (n=8 per genotype) is increased in mice lacking the mu opioid receptor. Data are presented as mean ± SEM. Genotype effect: one star p<0.05, two stars p<0.01, three stars p<0.001. See Figure S2 for more tests and parameters, and complete list of abbreviations.

neuronal activation across the two mouse populations (Table S3). Together, the data indicate that under defensive (marble burying) or conflict (NSF) conditions, mice lacking mu opioid receptors display exacerbated anxiety coherent with the autistic phenotype.

Besides anxiety, we used several tests to assess motor performance and coordination of *Oprm1*<sup>-/-</sup> mice, as these are often altered in autistic children (Green et al. 2009; Johnson and Myers 2007). Mice were trained to run on an accelerating rotarod for three trials a day during four consecutive days. *Oprm1*<sup>-/-</sup> mice stayed significantly shorter than control animals on the rod over sessions (Genotype:  $F_{1,10}=42.5$ ,  $p<0.001$ ; Gender:  $F_{1,10}<1$ , NS; Session:  $F_{3,30}=6.8$ ,  $p<0.01$ ; Trial:  $F_{2,20}=25.0$ ,  $p<0.001$ ; Genotype x Session:  $F_{3,30}=5.2$ ,  $p<0.01$ ; Genotype x Trial:  $F_{2,20}=5.2$ ,  $p<0.05$ ) (Figure 2f). Similarly, in the string test, these animals needed more time to grasp the string with a third paw (Genotype:  $F_{1,26}=6.7$ ,  $p<0.05$ ; Gender:  $F_{1,26}=1.5$ , NS), although their forelimb muscular strength was unchanged in the grip test (Figure S2b,c). Footprint analysis revealed an increased variability of pace length (sigma) in *Oprm1*<sup>-/-</sup> mice (Genotype:  $F_{1,27}=6.0$ ,  $p<0.01$ ; Gender:  $F_{1,27}<1$ , NS) (Figures 2g and S2d). These data demonstrate impaired motor coordination in mutant animals.

Finally, numerous autistic patients suffer from increased susceptibility to seizures (Robinson 2012). In agreement with earlier studies showing lowered seizure thresholds in response to several convulsant drugs in *Oprm1*<sup>-/-</sup> mice (Grecksch et al. 2004; Jang et al. 2001), we found that pentylenetetrazole (37.5 and 50 mg/kg) induced more severe convulsions in mutants as compared to *Oprm1*<sup>+/+</sup> animals (37.5 mg:  $H_{1,20}=11.6$ ,  $p<0.001$ ; 50 mg:  $H_{1,20}=10.0$ ,  $p<0.01$ ; no gender effect) (Figures 2h and S2e).

## **Behavioral deficits in *Oprm1*<sup>-/-</sup> mice result from the null mutation rather than parental care**

We assessed the impact of parental rearing on observed behavioral deficits by performing time-mated cross-fostering of *Oprm1*<sup>-/-</sup> mice and *Oprm1*<sup>+/+</sup> controls (Peca et al. 2011). Cross-fostered mutant animals showed impaired direct social interaction, stereotypic behavior and anxiety, but not nesting abilities, similar to those observed in *Oprm1*<sup>-/-</sup> mice nurtured by mutant parents (Figure S3). These behaviors were not affected in *Oprm1*<sup>+/+</sup> mice bred by mutant parents.

## ***Oprm1*<sup>-/-</sup> mice show modified transcription of several candidate genes for autism**

Multiple genes have been associated to the etiopathology of autism (Ecker et al. 2012a; Insel 2010; State and Levitt 2011). We quantified transcription levels of a selection of these genes in *Oprm1*<sup>+/+</sup> and *Oprm1*<sup>-/-</sup> animals across four brain regions critical for emotional/cognitive processes and motor control: the PFC, CPu, NAc and CeA, using a qRT-PCR approach (Figure 3a; Figure S4a, Table S4). We detected changes in the expression of genes coding for the adhesion and scaffold proteins neuroligins (*Nlgn1* and *Nlgn2*) and SHANK3 (*Shank3*). Transcriptional levels were also modified for the genes coding transporters of norepinephrine (NE, *Slc6a2*), dopamine (DA, *slc6a3*) and serotonin (5HT, *slc6a4*), as well as several receptors including beta3 subunit of GABAA receptors (*Gababrb3*) and 5HT2a serotonin and D2 dopamine receptors. The most dramatic changes in expression were observed for the genes coding the neuropeptides CRH and oxytocin. Finally, dysfunction of metabotropic glutamate receptors mGluR5 has received major attention as putative causative mechanism in fragile X syndrome and ASD (Carlson 2012). Expression of *Grm5*, coding these receptors, was not modified in *Oprm1*<sup>-/-</sup> mice; however expression of *Homer3*, coding a key molecular interactor of mGluR5 receptors, was significantly down-regulated. Transcriptional

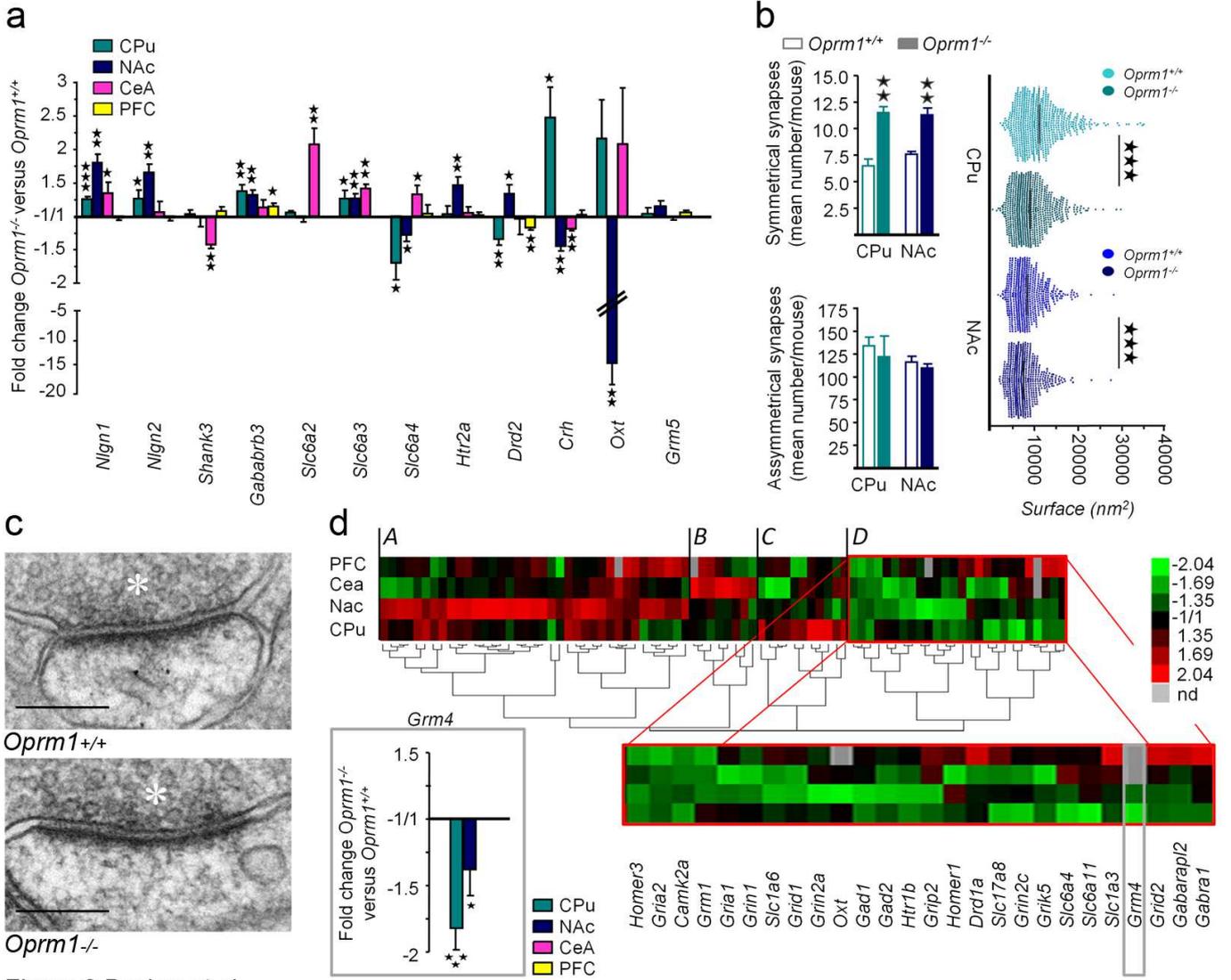


Figure 3 Becker et al.

**Figure 3. *Oprm1*<sup>-/-</sup> mice display transcriptional modifications and abnormal striatal synapses.**

(a) Transcription of multiple candidate genes for autism is modified in mutant animals across four brain regions (see details in Table S3). (b) *Oprm1*<sup>-/-</sup> mice display abnormalities in the number of striatal symmetrical synapses (left panels, 120 frames per mouse at 40,000x magnification). Striatal asymmetrical synapses show reduced surface of postsynaptic densities (right panel). (c) Representative microphotographs (40,000x) illustrate morphology of striatal asymmetrical synapses in *Oprm1*<sup>+/+</sup> and *Oprm1*<sup>-/-</sup> mice. Scale bar: 200 nm. Asterisk: presynaptic element. (d) Clustering analysis of gene expression data classifies genes in four clusters (A-D). Cluster (D) gathers genes mostly involved in glutamatergic signaling that all show lowered mRNA levels in the caudate putamen and nucleus accumbens of *Oprm1*<sup>-/-</sup> mice, among which *Grm4* (framed), coding mGluR4 glutamate receptors, was detected only in these regions. Data in (a) and (b) are presented as mean  $\pm$  SEM. Genotype effect: one star  $p < 0.05$ , two stars  $p < 0.01$ , three stars  $p < 0.001$ . CeA: central nucleus of the amygdala, CPu: caudate putamen, NAc: nucleus accumbens, PFC: prefrontal cortex. See also Figure S4.

modifications observed in mutants were region-specific, prominent in the CPu and NAc. Inactivation of the mu opioid receptor gene, therefore, alters expression of several candidate genes for ASD, mainly in striatal regions.

### **Mutant mice display abnormal striatal synapses, and modified transcription of GABA and glutamate signaling genes**

Abnormal basal ganglia function has been proposed as a key feature of autism (Di Martino et al. 2011; Kohls et al. 2012). In this study, deficient social behavior and motor clumsiness associated to stereotypic behavior in *Oprm1*<sup>-/-</sup> animals point towards ventral and dorsal striatal dysfunction. We used electron microscopy to assess striatal synaptic morphology and observed an increased number of symmetrical synapses in both CPu and NAc of *Oprm1*<sup>-/-</sup> mice (Genotype:  $F_{1,20}=67.6$ ,  $p<0.001$ ; Structure:  $F_{1,20}<1$ , NS), suggesting that inhibitory (GABAergic/glycinergic) local micro-circuitry is denser in these animals. The number of asymmetrical (glutamatergic) synapses was similar in mutants and controls; however, morphological analysis showed significant reduction in the area (CPu:  $F_{1,1505}=81.3$ ,  $p<0.001$ ; NAc:  $F_{1,1209}=19.1$ ,  $p<0.001$ ), but not length, of post-synaptic densities (PSDs; Figures 3b,c and S4b,c). These data strongly suggest that lack of mu opioid receptors alters the normal development of striatal synapses.

Recent studies have revealed abnormal GABA and glutamate metabolism in individuals with autism, notably in striatal regions for the latter (Horder et al. 2013; Rojas et al. 2013). To test for altered signaling within these two major neurotransmitter systems, we performed comprehensive expression analysis of critical GABA and glutamate gene players across previous four brain regions (Table S4). We then identified groups of genes sharing similar expression profiles, using cluster analysis of all qRT-PCR data (Figures 3d and S4). Hierarchical clustering organized gene expression in five main clusters. Clusters (A) (B) and (C) grouped genes with high transcription levels in the NAc, CeA or CPu of mutant mice,

respectively. Sixteen genes in clusters (A) and (C) code GABAergic receptors/receptor subunits and transporters (Figure S4d), consistent with increased number of striatal symmetrical synapses in knockout animals. Cluster (D) retained our attention for gathering genes (zoomed in Figure 3d) with consistent lowered mRNA levels in the CPu and NAc of *Oprm1*<sup>-/-</sup> mice, as compared to controls. Remarkably, fourteen genes in cluster (D) code glutamate receptors/receptor subunits and interacting proteins present in PSDs, further supporting electron microscopy data showing reduced PSD surface in mutants. Three major actors of the glutamate pathway, *Gria2*, *Homer3* and *Camk2a*, showed low transcript levels across all tested brain regions, suggesting widespread alterations of glutamate signaling. Among genes coding metabotropic glutamate receptors, only *Grm4* displayed statistically significant down-regulated expression and clustered in (D). Together, results suggest that mu opioid receptor gene knockout alters both GABA and glutamate signaling in the basal ganglia.

### **The positive mGluR4 allosteric modulator VU0155041 alleviates autistic symptoms in *Oprm1*<sup>-/-</sup> mice: comparison with the atypical antipsychotic risperidone**

Our qRT-PCR analysis revealed decreased transcription of *Grm4*, coding metabotropic glutamate receptors mGluR4, in the CPu and NAc of *Oprm1*<sup>-/-</sup> animals (framed in Figure 3d). We reasoned that restoring mGluR4 activity in mutant mice using a positive allosteric modulator (PAM) may relieve behavioral deficits in these animals. We compared efficiency of a chronic treatment (7-17 days; Figure S5a) with VU0155041, a mGluR4 PAM (5 mg/kg), or risperidone (0.2 mg/kg) in improving autistic symptoms. Chronic treatment was chosen for better translational value, and to match published conditions of risperidone administration in mice (Penagarikano et al. 2011). We used risperidone as a reference, since atypical antipsychotics remain the only approved drugs for symptomatic treatment of ASDs. Risperidone acts at D2 and 5HT2a receptors and alleviates aggression and repetitive/self-

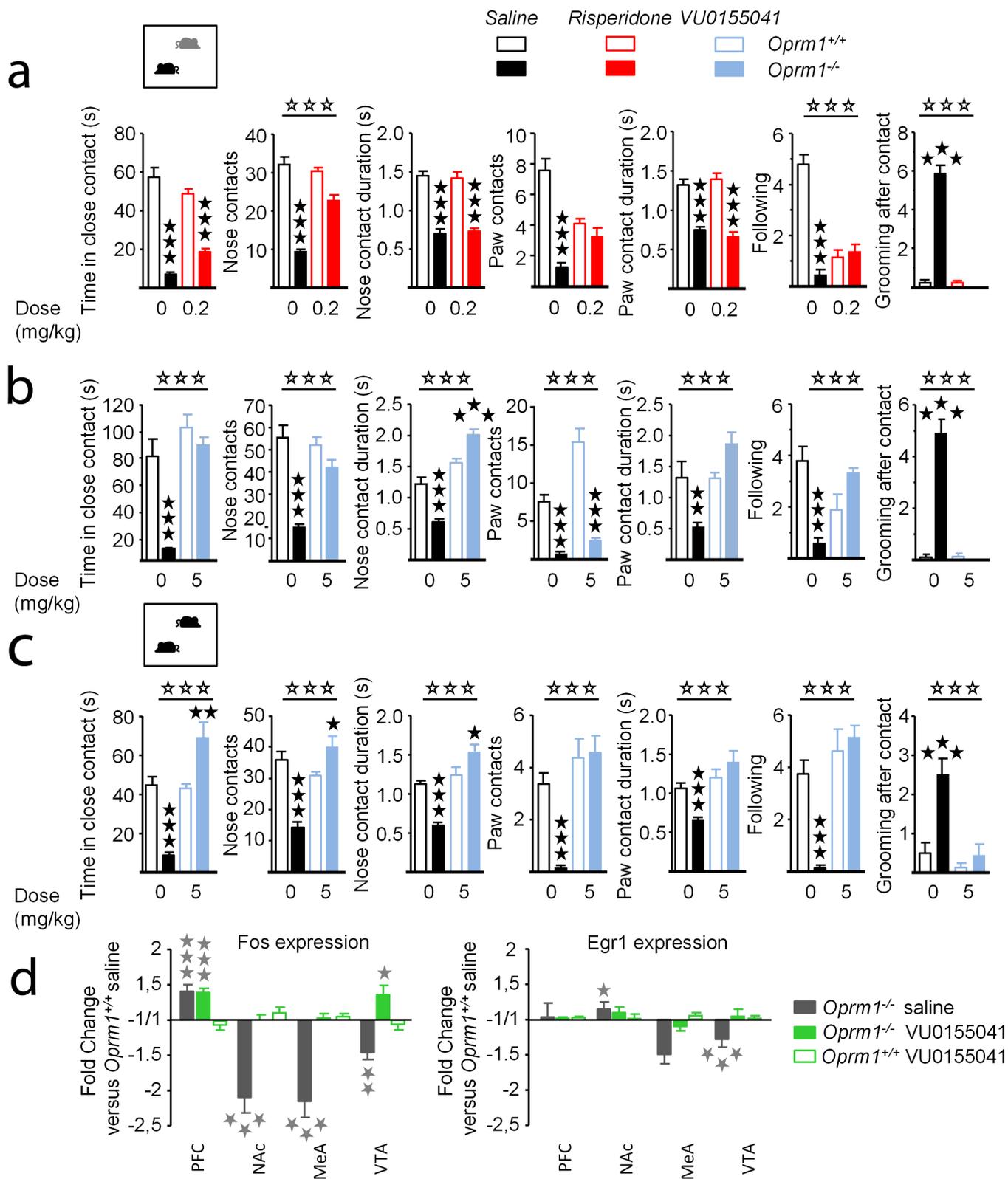
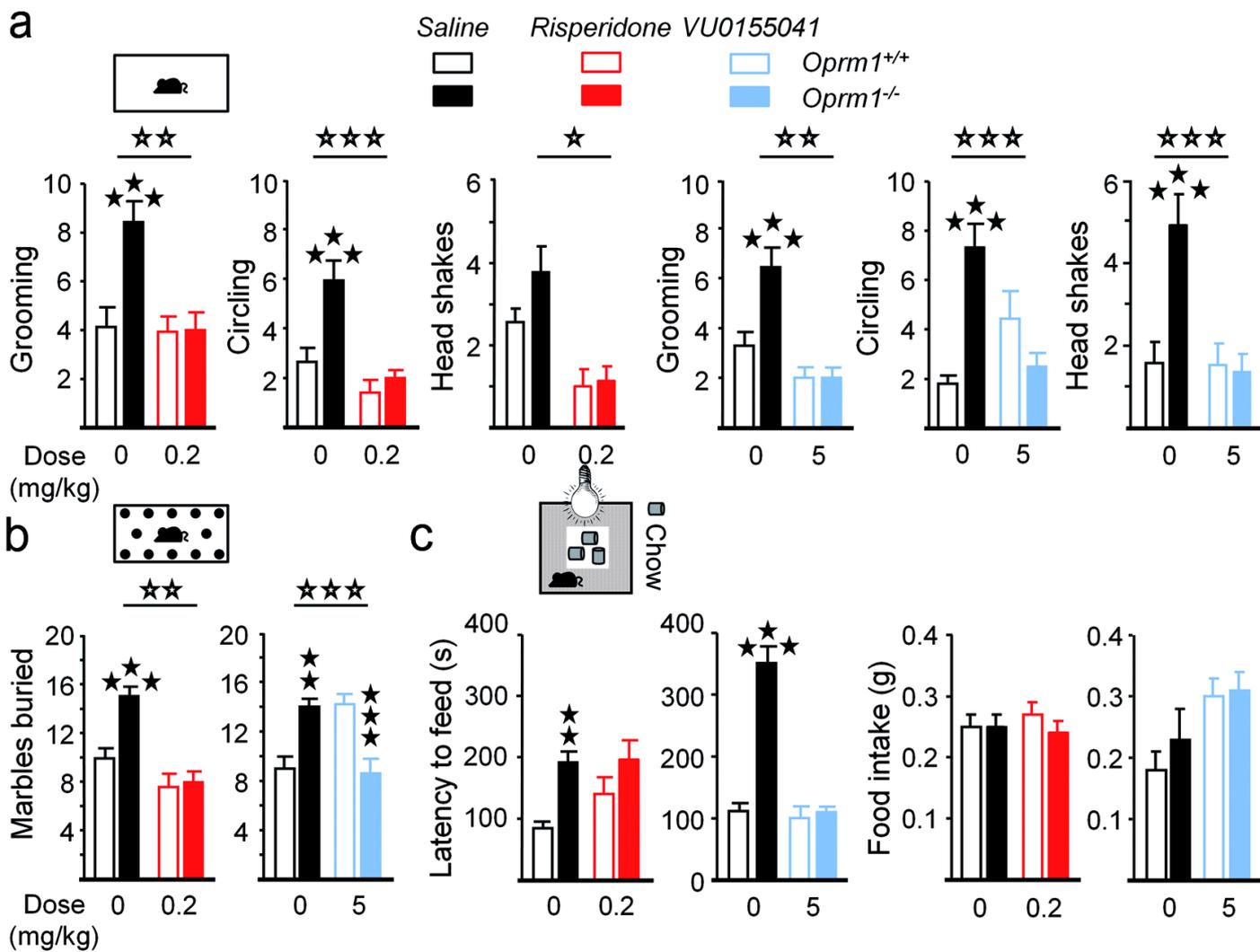


Figure 4 Becker et al.

**Figure 4. VU0155041, a positive allosteric modulator of mGluR4 glutamate receptors, and not the antipsychotic risperidone, restores initiation of social interaction in *Oprm1*<sup>-/-</sup> mice.** (a) When a naïve wild-type social partner is used in the dyadic interaction test, chronic risperidone (0.2 mg/kg) restores normal frequency of nose and paw contacts as well as grooming episodes, but fails to increase the time spent in close contact, the duration of nose and paw contacts and the number of following episodes in mutant animals (n=14 per genotype and treatment). (b) Chronic treatment with VU0155041 (5 mg/kg) improves all these parameters except the number of following episodes, and increases the occurrence of paw contacts in controls (9-10 per genotype and treatment). (c) Using a paradigm in which each experimental mouse encounters a genotype and treatment-matched animal confirms the efficiency of VU0155041 to normalize or even facilitate social interaction in mutant mice, including the frequency of following episodes (n=7-8 per genotype and treatment). (d) Chronic VU0155041 administration normalizes deficient *C-fos* expression following social interaction in the NAc, MeA and VTA as well as deficient *Egr1* expression in the VTA of *Oprm1*<sup>-/-</sup> mice. Data are presented as mean ± SEM. Open stars: treatment effect; black stars: genotype x treatment interaction (post-hoc: Newman-Keules test); grey stars: comparison with saline-treated *Oprm1*<sup>+/+</sup> mice. One star p<0.05, two stars p<0.01, three stars p<0.001.

injurious behavior, but fails to significantly improve social abilities (Maher and Theodore 2012).

Risperidone and VU0155041 treatments demonstrated beneficial effects in the social interaction test by increasing the time spent in close contact and frequency of nose and paw contacts in mutants. Risperidone, however, failed to increase the duration of close contacts, while VU0155041 normalized or even increased this duration. Both treatments reduced grooming, especially after social contact (statistics in Tables S5 and S6), when treated animals interacted with a naïve WT conspecific (Figure 4a,b). Risperidone reduced activity of *Oprm1*<sup>+/+</sup> control mice in our strain, as shown by decreased paw contacts and following in this test, confirmed by reduced horizontal activity and coherent with decreased striatal DA levels (Figure S5b,c; Table S7), in agreement with well described adverse sedative effects of atypical antipsychotics (Maher and Theodore 2012). In contrast, VU0155041 increased paw contacts and horizontal activity in both *Oprm1*<sup>+/+</sup> and *Oprm1*<sup>-/-</sup> lines (Figure S5d). To assess whether risperidone and VU0155041 treatments similarly restore initiation of social interaction despite opposing effects on activity, we performed the social interaction test with each experimental animal encountering a gender-, genotype- and treatment-matched conspecific (Figure S5g). No interactions could be observed between stimulus and test mice under risperidone, for both *Oprm1*<sup>+/+</sup> and *Oprm1*<sup>-/-</sup> lines, whereas VU0155041 fully restored social behavior in mutant mice with no effect in *Oprm1*<sup>+/+</sup> animals (Figure S5h; statistics in Table S8). Finally, we assessed the effects of mGluR4 PAM treatment on neuronal activity induced by social exposure by measuring transcript levels of two immediate early genes, *C-fos* and *Egr1*, following direct social interaction (second paradigm, Figure S5g). We used qRT-PCR for high detection sensitivity, and focused on brain regions involved in the control of reward processes and/or social behavior (PFC, NAc, VTA and MeA) (van Kerkhof et al. 2013; Weathington et al. 2012). We confirmed that under these conditions chronic



**Figure 5. VU0155041 alleviates secondary symptoms of autism in *Oprm1*<sup>-/-</sup> mice more efficiently than risperidone.**

(a) Both risperidone (left) and VU0155041 (right) treatments suppress motor stereotypies. (b) In the marble burying test, risperidone and VU0155041 normalize stereotyped burying in mutant mice; VU0155041, however, increases anxiety-induced burying in *Oprm1*<sup>+/+</sup> mice. (c) Latencies to feed in the NSF do not significantly differ between *Oprm1*<sup>+/+</sup> and *Oprm1*<sup>-/-</sup> mice under risperidone treatment, while VU0155041 reverses the high latency to eat of *Oprm1*<sup>-/-</sup> mice to control levels. Food intake remains unchanged. Data are presented as mean ± SEM. Open stars: treatment effect; solid stars: genotype x treatment interaction (post-hoc: Newman-Keules); One star p<0.05, two stars p<0.01, three stars p<0.001. See also Figure S5.

VU0155041 administration alleviates deficient social behavior in mutant mice (Figure 4c). Concomitantly, this treatment normalized the decreased expression of *C-fos* in the NAc, VTA and MeA of mutant animal after social interaction. Similarly, VU0155041 normalized or tended to normalize decreased expression of *Egr1* in the VTA and MeA, respectively, and brought increased *Egr1* transcription in the NAc back to wild-type levels in *Oprm1*<sup>-/-</sup> mice (Figure 4d). These effects were not observed at the level of PFC. Thus mGluR4 PAM treatment, when administered to mutant mice, restored neuronal reactivity induced by social exposure in specific brain regions associated to reward and social behavior.

Neither risperidone nor VU0155041 improved nesting scores in mutant animals (Figure S5e). In contrast, both suppressed stereotyped grooming and circling (Figures 5a and S5f) and normalized marble burying in *Oprm1*<sup>-/-</sup> mice. VU0155041, however, increased stereotypic marble burying in *Oprm1*<sup>+/+</sup> mice (Figure 5b). Risperidone had no detectable effect in the NSF; in contrast, VU0155041 restored a short latency to eat in mutant animals (Figure 5c; statistics in Tables S5 and S6). Compared to risperidone, the mGluR4 PAM was thus more efficient in alleviating social deficits and anxiety in *Oprm1*<sup>-/-</sup> mice, with no depressive effect on general activity. To our knowledge, this is the first evidence that mGluR4 represents a promising pharmacological target in the context of ASDs.

## Discussion

Altogether, our data demonstrate that *Oprm1* deletion is sufficient to produce a comprehensive spectrum of behavioral, neuroanatomical, biochemical and genetic landmarks of ASDs. Behaviorally, marked social interaction deficits in mutant animals extend previous findings of reduced maternal attachment and altered social and communication skills in these mice (Cinque et al. 2012; Moles et al. 2004; Wohn et al. 2011) to definitely demonstrate that mu opioid receptors are essential for establishing appropriate social behavior. Stereotyped and

perseverative behaviors detected in *Oprm1*<sup>-/-</sup> mice further complete autistic-like core symptoms in these animals. In addition, mutant mice show multiple comorbid symptoms of ASD, including aggressiveness, exacerbated anxiety, motor clumsiness and increased susceptibility to seizures. Together with previously described impairments in spatial learning (Jamot et al. 2003), lowered nociceptive thresholds (Gaveriaux-Ruff and Kieffer 2002) and reduced gastrointestinal motility (Roy et al. 1998), mice lacking mu opioid receptors recapitulate the broadest autistic syndrome, including comorbid signs (Argyropoulos et al. 2013; Veenstra-VanderWeele and Blakely 2012) but not gender bias (usually not tested in mouse models), ever described in preclinical research. *Oprm1*<sup>-/-</sup> mice thus display remarkable face validity as an animal model of this pathology.

Social interactions have been demonstrated to be intrinsically rewarding (Neuhaus et al. 2010; Trezza et al. 2010). Interestingly, imaging and psychophysical studies have provided evidence for reward dysfunction in autistic patients (Dichter et al. 2012; Kohls et al. 2012). These data are coherent with a recently emerged reward/motivation deficiency theory of autism (Chevallier et al. 2012; Dichter and Adolphs 2012) which proposes that disrupted social interest in ASD patients results from early deficits in their motivation for attending social stimuli, as well as enjoying and prolonging reciprocal social interactions. In humans, neural sensitivity to social rejection (Way et al. 2009) and social hedonic capacity (Troisi et al. 2011) show significant association with a common variant (A118G) of *Oprm1*, and social acceptance activates mu opioid receptors in the NAc (Hsu et al. 2013), suggesting that mu opioid receptors are involved in social reward. Mu opioid receptors indeed play a critical role in mediating reward processes (Le Merrer et al. 2009). Interestingly, *Oprm1*<sup>-/-</sup> mice show altered social place preference (Cinque et al. 2012) and blunted induction of *C-fos* and *Egr1* expression following social challenge in brain regions controlling reward processes and social recognition (NAc, VTA, MeA). These data suggest that social reward is compromised in

these animals, and thus substantiate the reward deficiency theory. Reduced mu opioid receptor function in early life, therefore, could represent a major molecular mechanism underlying reward and social behavior deficits in ASDs. Altered mu opioid receptor signaling would result either from invalidating mutations of *Oprm1*, as identified in several patients with ASD (SFARIgene<sup>2.0</sup> database <https://gene.sfari.org/autdb/Welcome.do>), or from genetic or environmental factors that hamper mu opioid receptor activity. Alterations in other neurotransmitter systems may also contribute, and the profound decrease in *Oxt* gene expression in the NAc of *Oprm1*<sup>-/-</sup> mice is most striking. Remarkably, oxytocin release in this region has been demonstrated to play a key role in social reward (Dolen et al. 2013). Moreover, this neuropeptide has been involved in the etiology of autism (Insel 2010), and oxytocin treatment demonstrated beneficial effects on social skills in ASD patients (Andari et al. 2010). Future studies will clarify mu opioid/oxytocin interactions and determine whether low oxytocin activity may result from deficient mu opioid receptor signaling.

At cellular and molecular level, mutant mice also show most features characterizing ASDs in animal and clinical research. *Oprm1*<sup>-/-</sup> mice exposed to a conflict situation showed increased and decreased neuronal activation in anxiety- and reward-associated brain structures, respectively, mimicking activation patterns observed in autistic patients (Dichter et al. 2012; Kohls et al. 2012). High 5HT and NE concentrations in the dorsal raphe of mutant mice (Table S5) parallel high blood levels for these two monoamines reported in autistic subjects (Lam et al. 2006). Altered expression of monoamine signaling genes (*Slc6a2*, *Slc6a4*, *Htr2a*, *Maoa*) is in line with findings of ASD behavioral features elicited by increased monoaminergic activity (Bortolato and Shih 2011). Mutant mice also showed altered expression for multiple genes associated to ASDs (Ecker et al. 2012a; Insel 2010; State and Levitt 2011). Finally, we collected anatomical and molecular evidence for altered glutamatergic neurotransmission, a feature common to several mouse models of ASD

(Carlson 2012; Peca et al. 2011; Won et al. 2012) and supported by genetic studies (Kelleher et al. 2012; Sato et al. 2012). Notably, expression of multiple genes coding major players of glutamate neurotransmission was down-regulated in striatal regions.

We next tested therapeutic utility of *Oprm1*<sup>-/-</sup> mice in the context of ASDs. Metabotropic glutamate receptors have received major attention as potential therapeutic targets for the treatment of ASDs (Carlson 2012). Transcriptome analysis identified expression of *Grm4*, coding metabotropic glutamate receptors mGluR4, as significantly down-regulated in the striatum of mutant animals. *Grm4* shows a discrete pattern of mRNA expression in the brain, including striatal regions (Testa et al. 1994). High levels of presynaptic mGluR4 receptors are transported to nigral and pallidal output regions (Bradley et al. 1999; Corti et al. 2002) where they modulate GABA release. Activation of these receptors notably reduces GABAergic striatopallidal neurotransmission, with beneficial effects on dyskinesia/akinesia in animal models of Parkinson's disease (Amalric et al. 2013). Moreover, PAMs of mGluR4 receptors decrease anxiety-like behavior in the marble burying and Vogel's conflict tests (Duvoisin et al. 2011; Slawinska et al. 2013). We thus reasoned that restoring mGluR4 activity in mutant mice using such compound may ameliorate behavioral deficits in these animals. We found that chronic treatment with the PAM VU0155041 was more efficient than the antipsychotic risperidone in improving autistic-like symptoms in *Oprm1*<sup>-/-</sup> mice, especially regarding social behavior, without sedative side effects. Interestingly, a chronic moderate dose of risperidone reduced striatal dopamine levels in our mice. Therefore, common beneficial effects of risperidone and VU0155041 on autistic-like deficits in our mouse model, despite their distinct molecular targets, may lie in their shared ability to moderate striatopallidal output, an intriguing novel hypothesis in the field. Remarkably, VU0155041 treatment in *Oprm1*<sup>-/-</sup> mice normalized immediate early gene expression induced by social exposure in several brain regions processing reward and social

recognition, suggesting that facilitation of mGluR4 signaling was able to restore social reward in these animals.

In conclusion, this study substantiates the notion that ASDs could be considered an extreme case of early-onset deficient reward processes, and proposes facilitation of mGluR4 receptor activity as a promising novel therapeutic strategy. Future investigations will aim at better delineating the impact of mu opioid receptor deletion across brain regions and along the life span as a trigger for autistic syndrome, using local or time-controlled manipulations of gene expression.

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### **Author contributions**

J.L.M., J.A.J.B. and B.L.K. designed the experiments. J.L.M. and J.A.J.B. performed and analysed behavioural, immunohistochemical, qRT-PCR and pharmacological experiments. D.C. performed and analysed HPLC dosage of monoamine levels in brain tissue. C. S. and Y. S. performed and analysed electron microscopy experiments. J.L.M., J.A.J.B., Y.S. and B.L.K.

interpreted the results. J.L.M., J.A.J.B. and B.L.K. wrote the article. J.L.M. and B.L.K. contributed equally to this work. All authors discussed the results and commented on the manuscript.

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