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Autistic-Like Syndrome in Mu Opioid Receptor Null Mice is Relieved by Facilitated mGluR4 Activity

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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*$^{-/-}$) 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*$^{-/-}$ 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 signaling is sufficient to trigger a comprehensive autistic syndrome, maybe through blunted social reward processes, and this mouse model opens promising avenues for therapeutic innovation.
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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 identified over 280 candidate vulnerability genes for ASD (SFARIgene[2.0] 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*$^{-/-}$) 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

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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 predicts the desire for social interaction (Hsu *et al*, 2013). Finally, genetic studies have detected discrete invalidating mutations of the *Oprm1* gene in some ASD patients (SFARIgene$^{2.0}$ database), suggesting that such invalidation may contribute to the development of an autistic syndrome, and providing construct validity for the *Oprm1*$^{-/-}$ 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.

MATERIALS AND METHODS

Subjects

Male and female *Oprm1*$^{+/+}$ and *Oprm1*$^{-/-}$ 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*$^{+/+}$ and *Oprm1*$^{-/-}$ pups were nurtured by *Oprm1*$^{+/+}$ and *Oprm1*$^{-/-}$ parents, respectively, except for cross-fostering experiment. Except otherwise stated, animals were group housed and maintained on a 12-h light/dark cycle (lights on at 070 hours) at controlled temperature ($21 \pm 1\,^{\circ}C$); 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 Materials 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 (NSF) tests. Motor function was assessed using the string and grip tests, skill motor learning task, and footprint analysis. Sensitivity to seizures was assessed by scoring penthylene tetrazole-induced convulsions. Experiments were performed on separate cohorts of naive animals (Figures 1 and 2), except for tests exploring motor function (string test, grip test, accelerating rotarod, footprint analysis, in this order), cross-fostering experiment (Supplementary Figure S3), and pharmacological experiments (Figures 4 and 5), where behavioral testing was performed in a battery (time lines in Supplementary Figures S3a and S5a,g). 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). Prefrontal cortex (PFC), caudate putamen (CPu), nucleus accumbens (Nac), central amygdala (CeA) and ventral tegmental area (VTA) were punched out, and medial amygdala (MeA) was dissected, out from 1-mm thick slices. Tissues were immediately frozen on dry ice and kept at $-80\,^{\circ}C$ 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 synthetized 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 Supplementary 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) at 0.2 mg/kg i.p. (Penagarikano *et al*, 2011) was administered 60 min before testing. VU0155041 (Tocris, Bristol, UK) at 5 mg/kg i.p. (Duvoisin *et al*, 2011) was injected 120 min before testing. When risperidone and VU0155041 effects were compared with the same vehicle-treated group, control animals were tested 60 min after vehicle injection. All compounds were administered in a volume of 10 ml/kg; vehicle was NaCl 0.9%.

Statistical Analyses

Statistical analyses were performed using the 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-Keuls *post-hoc* test, except for nesting and convulsion scores, for 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

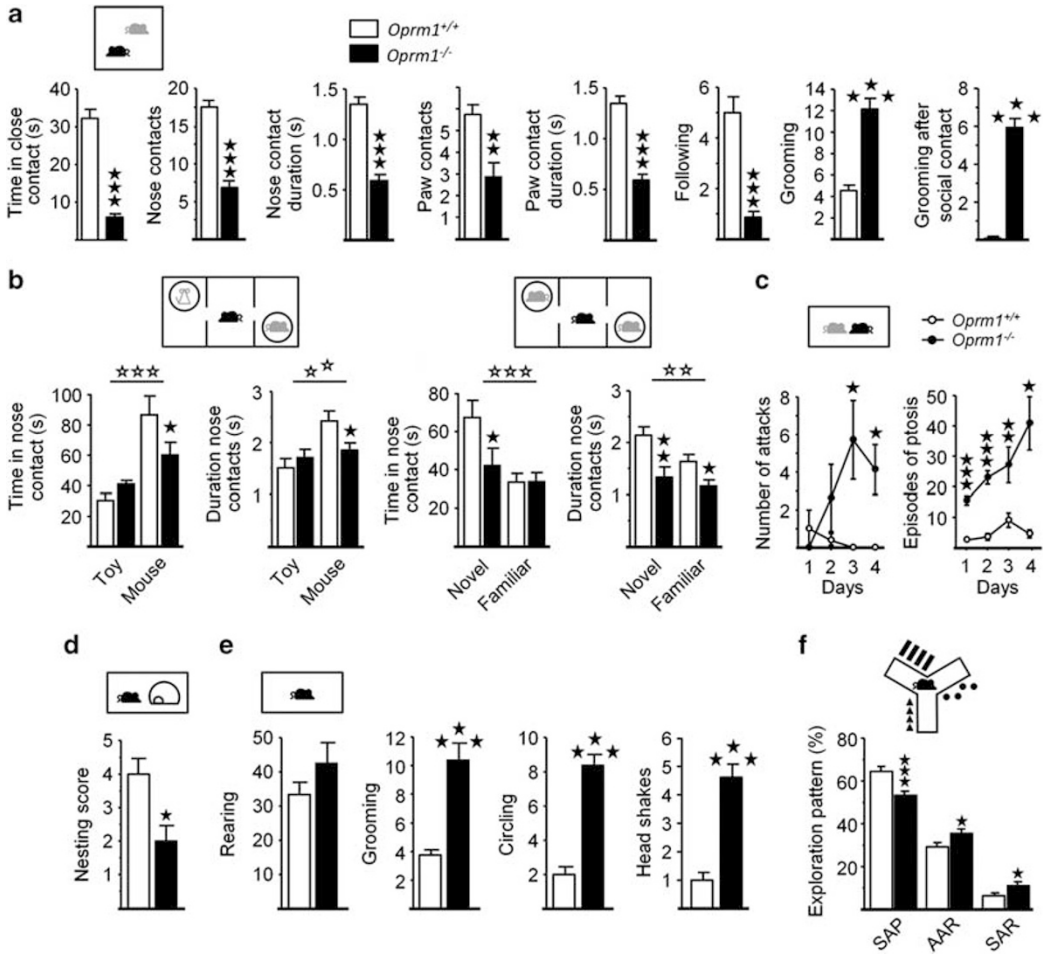

Figure 1 *Oprm1*$^{-/-}$ mice show core symptoms of autism. (a) In the direct social interaction test, *Oprm1*$^{-/-}$ animals show decreased time in close social contact, due to lower frequency and duration of nose and paw contacts. Following was decreased, whereas self-grooming, especially after social contact, was increased in mutants ($n = 8$ per genotype). (b) In the three-chamber task, WT controls but not *Oprm1*$^{-/-}$ 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*$^{-/-}$ animals are more aggressive in the resident-intruder test, with increased numbers of attacks and episodes of ptosis ($n = 8$ per genotype). (d) *Oprm1*$^{-/-}$ mice show impaired ability to build a nest ($n = 8$ per genotype). (e) *Oprm1*$^{-/-}$ 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*$^{-/-}$ 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 Supplementary Figure S1.

Fos count data. Loadings for each extracted principal component (PC) are quoted in Supplementary Table S2. 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 Materials and Methods.

RESULTS

Oprm1$^{-/-}$ 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*$^{-/-}$ mice using four tests. In the direct social interaction test, *Oprm1*$^{-/-}$ mice spent less time in close interaction with a naive wild-type (WT) conspecific compared with *Oprm1*$^{+/+}$ 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 (Figure 1a and Supplementary Figure S1a, statistics in Supplementary Table S3). In the three-chamber test, *Oprm1*$^{+/+}$ but not *Oprm1*$^{-/-}$ animals spent more time in nose contact with a conspecific *vs* a toy mouse during the interaction phase. When the toy mouse was replaced by a novel conspecific (recognition phase), *Oprm1*$^{+/+}$ but not *Oprm1*$^{-/-}$ mice spent more time interacting with this unfamiliar animal (Figure 1b and Supplementary Figure S1b, Supplementary Table S3). Defective recognition of a novel social partner did not result from general alteration of recognition abilities, as object recognition was preserved in mutant

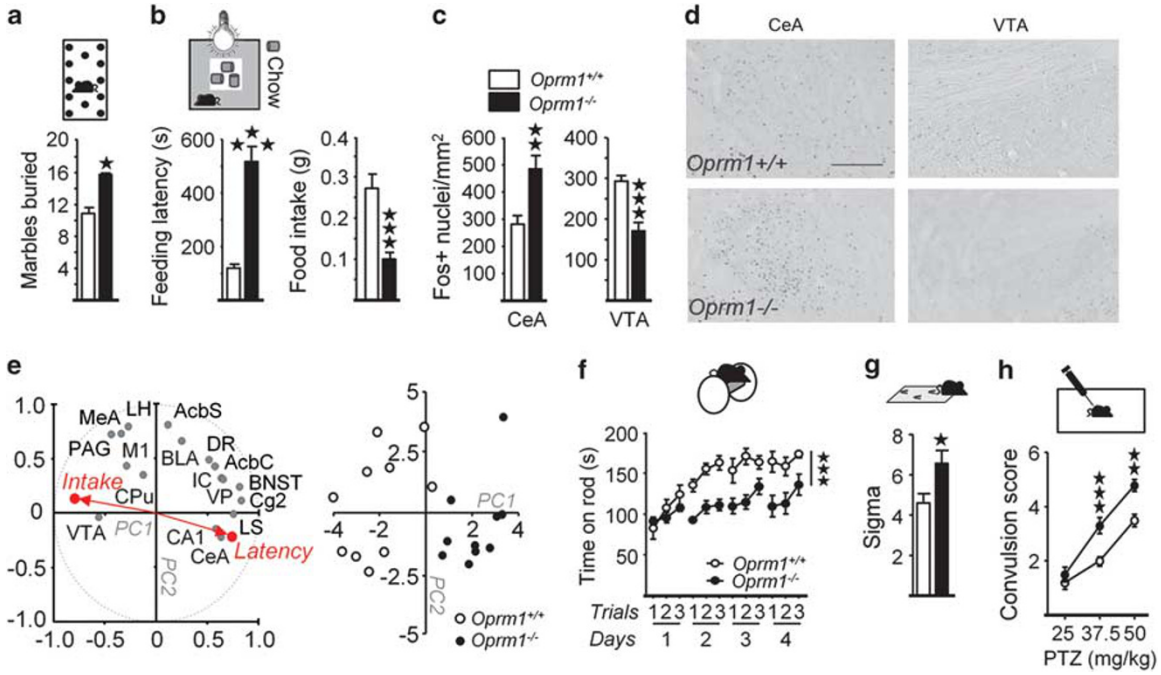

Figure 2 *Oprm1*$^{-/-}$ mice display multiple secondary symptoms of autism. (a) Defensive anxiety is higher in mutant animals, hiding more marbles than controls in the marble-burying test ($n=10$ per genotype). (b) Conflict anxiety is also increased, as shown by longer feeding latencies in the novelty-suppressed feeding (NSF) test ($n=10$ per genotype). (c, d) Increased anxiety of *Oprm1*$^{-/-}$ 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*$^{+/+}$ and *Oprm1*$^{-/-}$ individual mice are dissociated in the subjects' space (right panel). (f, g) Motor function is altered in mutant animals. *Oprm1*$^{-/-}$ 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 Supplementary Figure S2 for more tests and parameters and complete list of abbreviations.

animals (Supplementary Figure S1c), or from deficient olfactory perception, as shown in previous studies (Cinque *et al*, 2012; Moles *et al*, 2004). We observed that male *Oprm1*$^{-/-}$ 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 naive male was introduced in their home cage, daily for 4 days. In contrast with *Oprm1*$^{+/+}$ controls, mutant animals engaged in 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) (Figure 1c, Supplementary Figure S1d, and Supplementary Table S3). 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) (Supplementary Figure S1d). Thus aggressive behavior, a symptom often associated with ASDs, is increased in *Oprm1*$^{-/-}$ animals. Finally, these mice revealed impaired ability to build a nest in the nesting behavior test (Figure 1d, Supplementary Table S3). Together, these results indicate that adult *Oprm1*$^{-/-}$ 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*$^{-/-}$ mice displayed more frequent grooming, circling, and head shakes but not rearing or burying than *Oprm1*$^{+/+}$ animals (Figure 1e and Supplementary Figure 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 and reduced behavioral flexibility in *Oprm1*$^{-/-}$ mice therefore completed autistic-like core symptoms in mutant mice.

Oprm1$^{-/-}$ 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 with 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 NSF test, which tackle 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*$^{-/-}$ 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 with controls (Figure 2b). *Oprm1*$^{-/-}$ 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 with *Oprm1*$^{+/+}$, *Oprm1*$^{-/-}$ 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 and d; Supplementary Table S4). 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, Supplementary Table S2). Remarkably, projection in the subjects' space (Figure 2e right) clearly dissociated *Oprm1*$^{+/+}$ from *Oprm1*$^{-/-}$ individual mice, demonstrating distinct patterns of behavioral responses and neuronal activation across the two mouse populations (Supplementary Table S2). 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*$^{-/-}$ 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 4 consecutive days. *Oprm1*$^{-/-}$ 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 × Session: $F_{3,30}=5.2$, $p<0.01$; Genotype × 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 (Supplementary Figure S2b and c). Footprint analysis revealed an increased variability of pace length (sigma) in *Oprm1*$^{-/-}$ mice (Genotype: $F_{1,27}=6.0$, $p<0.01$; Gender: $F_{1,27}<1$, NS) (Figure 2g and Supplementary Figure 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*$^{-/-}$ 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 with *Oprm1*$^{+/+}$ 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) (Figure 2h and Supplementary Figure S2e).

Behavioral Deficits in *Oprm1*$^{-/-}$ 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*$^{-/-}$ mice and *Oprm1*$^{+/+}$ 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*$^{-/-}$ mice nurtured by mutant parents (Supplementary Figure S3). These behaviors were not affected in *Oprm1*$^{+/+}$ mice bred by mutant parents.

Oprm1$^{-/-}$ 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*$^{+/+}$ and *Oprm1*$^{-/-}$ 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; Supplementary Figure S4a, Supplementary Table S5). 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*$^{-/-}$ mice; however, expression of *Homer3*, coding a key molecular interactor of mGluR5 receptors, was significantly downregulated. Transcriptional 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 with stereotypic behavior in *Oprm1*$^{-/-}$ animals point towards ventral and dorsal striatal dysfunction. We used electron microscopy to assess striatal synaptic morphology and observed an increased

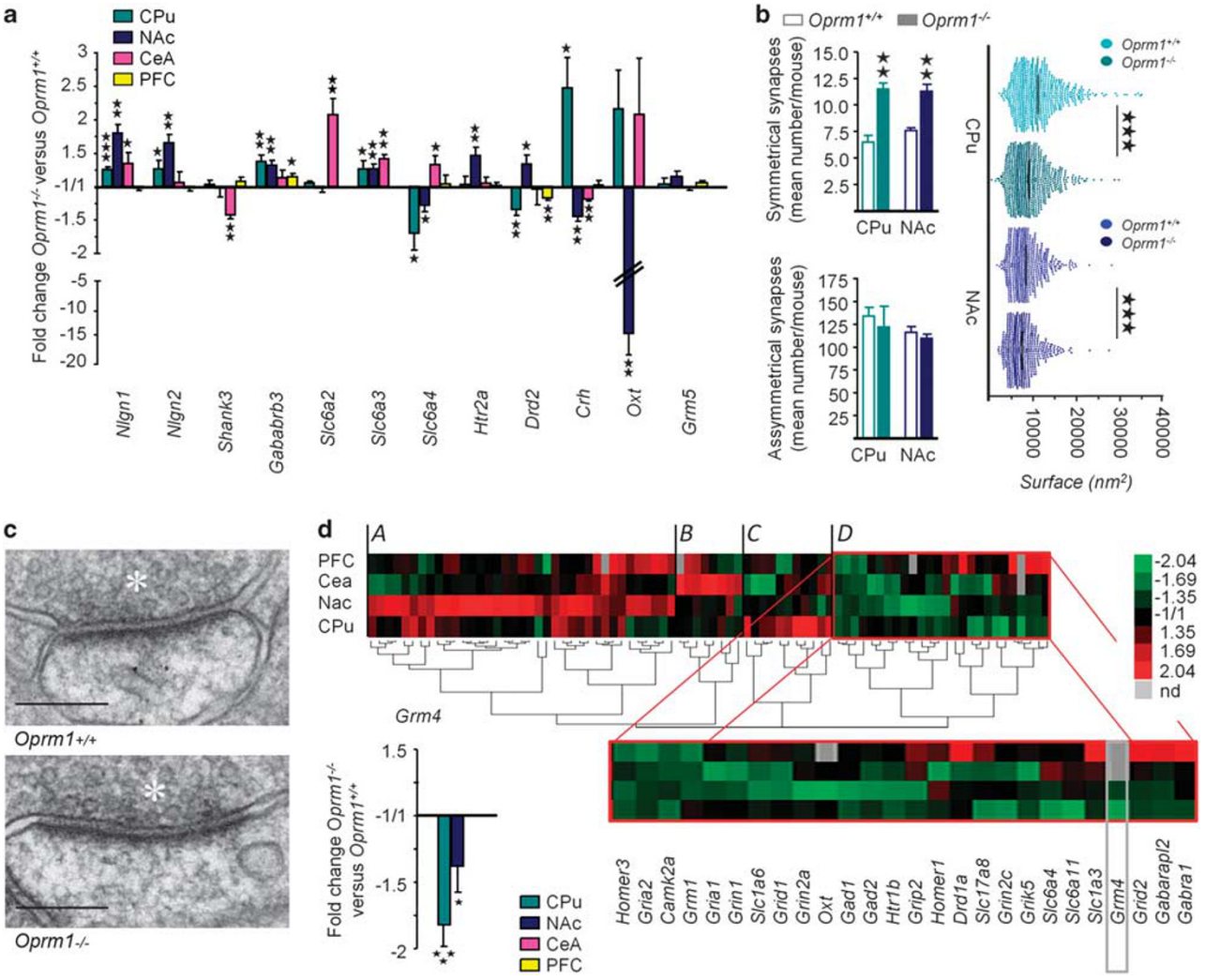

Figure 3 *Oprm1* $^{-/-}$ mice display transcriptional modifications and abnormal striatal synapses. (a) Transcription of multiple candidate genes for autism is modified in mutant animals across four brain regions (see details in Supplementary Table S3). (b) *Oprm1* $^{-/-}$ mice display abnormalities in the number of striatal symmetrical synapses (left panels, 120 frames per mouse at $\times$ 40 000 magnification). Striatal asymmetrical synapses show reduced surface of postsynaptic densities (right panel). (c) Representative microphotographs ($\times$ 40 000) illustrate morphology of striatal asymmetrical synapses in *Oprm1* $^{+/+}$ and *Oprm1* $^{-/-}$ 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* $^{-/-}$ mice, among which *Grm4* (framed), coding mGluR4 glutamate receptors, was detected only in these regions. Data in (a) and (b) are presented as mean ± 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 Supplementary Figure S4.

number of symmetrical synapses in both CPu and NAc of *Oprm1* $^{-/-}$ 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 postsynaptic densities (PSDs; Figure 3b and c and Supplementary Figure S4b and 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 (Supplementary Table S5). We then identified groups of genes sharing similar expression profiles, using cluster analysis of all qRT-PCR data (Figure 3d and Supplementary Figure 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 (Supplementary 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*$^{-/-}$ mice, as compared with controls. Remarkably, 14 genes in cluster (*D*) code glutamate receptors/receptor subunits and interacting proteins present in PSDs, further supporting electron microscopic data showing reduced PSD surface in mutants. Three major actors of the glutamate pathway, *Gria2*, *Homer3*, and *Camk2a*, showed low transcript levels across all the tested brain regions, suggesting widespread alterations of glutamate signaling. Among genes coding metabotropic glutamate receptors, only *Grm4* displayed statistically significant downregulated 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*$^{-/-}$ 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*$^{-/-}$ 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; Supplementary 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, as 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-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 Supplementary Tables S6 and S7), when treated animals interacted with a naive WT conspecific (Figure 4a and b). Risperidone reduced activity of *Oprm1*$^{+/+}$ 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 (Supplementary Figure S5b and c; Supplementary Table S8), 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*$^{+/+}$ and *Oprm1*$^{-/-}$ lines (Supplementary 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 (Supplementary Figure S5g). No interactions could be observed between stimulus and test mice under risperidone for both *Oprm1*$^{+/+}$ and *Oprm1*$^{-/-}$ lines, whereas VU0155041 fully restored social behavior in mutant mice, with no effect in *Oprm1*$^{+/+}$ animals (Supplementary Figure S5h; statistics in Supplementary Table S9). Finally, we assessed the effects of mGluR4 PAM treatment on neuronal activity induced by social exposure by measuring the transcript levels of two immediate early genes, *C-fos* and *Egr1*, following direct social interaction (second paradigm, Supplementary 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 VU0155041 administration alleviates deficient social behavior in mutant mice (Figure 4c and Supplementary Table S10). 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 WT levels in *Oprm1*$^{-/-}$ 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 with reward and social behavior.

Neither risperidone nor VU0155041 improved nesting scores in mutant animals (Supplementary Figure S5e). In contrast, both suppressed stereotyped grooming and circling (Figure 5a and Supplementary Figure S5f) and normalized marble burying in *Oprm1*$^{-/-}$ mice. VU0155041, however, increased stereotypic marble burying in *Oprm1*$^{+/+}$ 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 Supplementary Tables S6 and S7). Compared with risperidone, the mGluR4 PAM was thus more efficient in alleviating social deficits and anxiety in *Oprm1*$^{-/-}$ 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; Wohr *et al*, 2011) to definitely demonstrate that mu opioid receptors are essential for establishing appropriate social behavior. Stereotyped and perseverative behaviors detected in *Oprm1*$^{-/-}$ 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

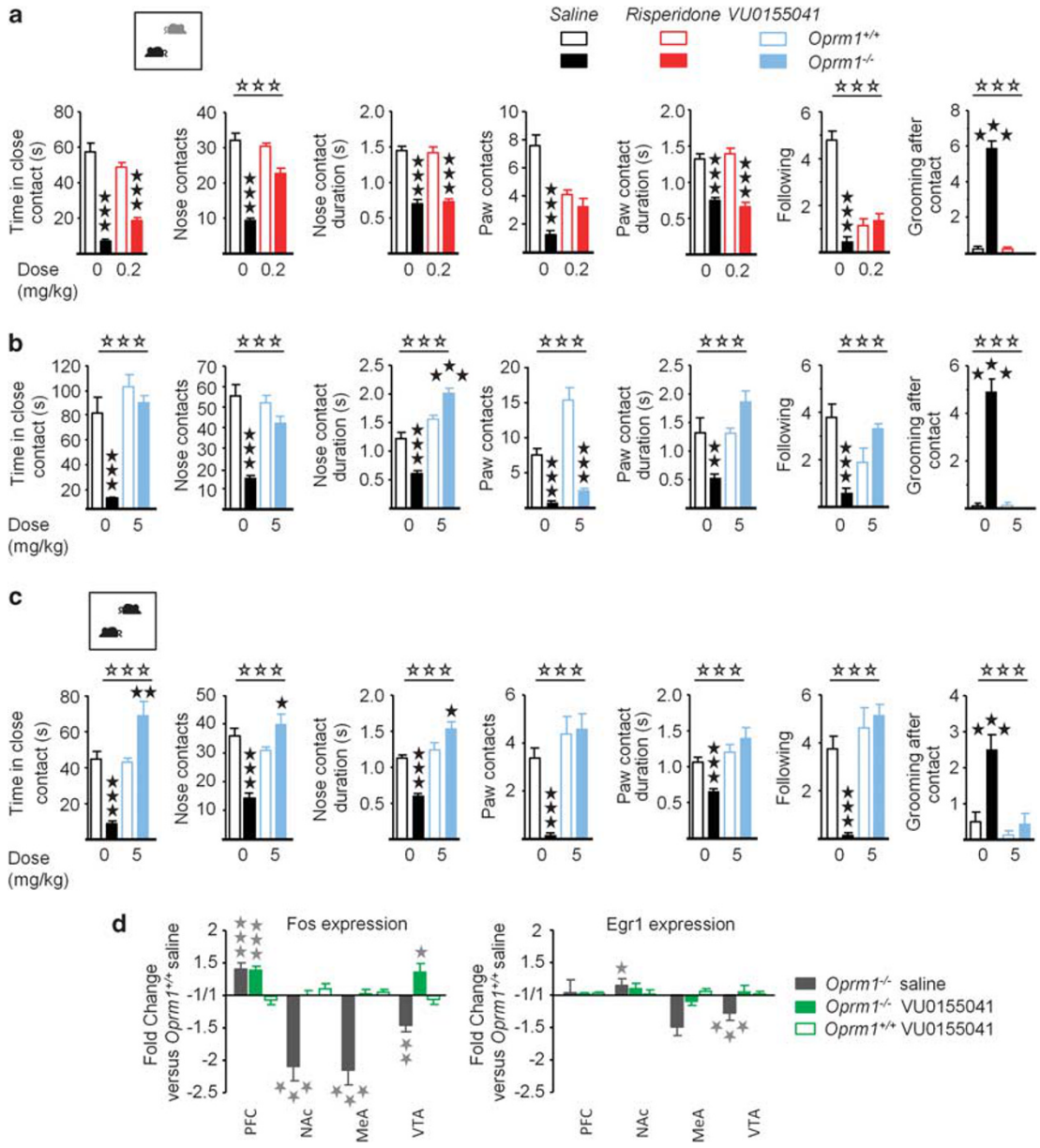

Figure 4 VU0155041, a positive allosteric modulator of mGluR4 glutamate receptors and not the antipsychotic risperidone, restores initiation of social interaction in *Oprm1*$^{-/-}$ mice. (a) When a naive wild-type social partner is used in the dyadic interaction test, chronic risperidone (0.2 mg/kg) 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 *Oprm*$^{-/-}$ mice. Data are presented as mean ± SEM. Open stars: treatment effect; black stars: genotype × treatment interaction (*post-hoc*: Newman–Keuls test); grey stars: comparison with saline-treated *Oprm1*$^{+/+}$ mice. One star $p < 0.05$, two stars $p < 0.01$, three stars $p < 0.001$.

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*$^{-/-}$ 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

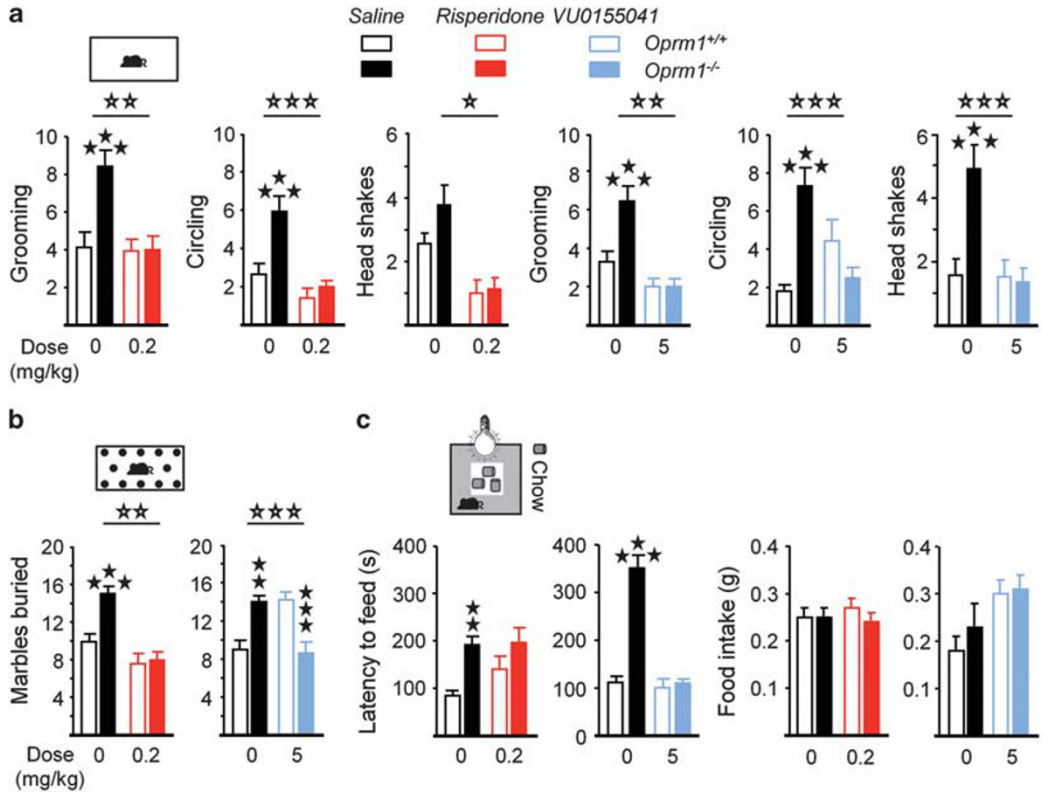

Figure 5 VU0155041 alleviates secondary symptoms of autism in *Oprm1*$^{-/-}$ mice more efficiently than risperidone. (a) Both risperidone (left) and VU0155041 (right) treatments suppress motor stereotypies. (b) In the marble burying test, risperidone and VU0155041 normalize stereotyped burying in mutant mice; VU0155041, however, increases anxiety-induced burying in *Oprm1*$^{+/+}$ mice. (c) Latencies to feed in the NSF do not significantly differ between *Oprm1*$^{+/+}$ and *Oprm1*$^{-/-}$ mice under risperidone treatment, while VU0155041 reverses the high latency to eat of *Oprm1*$^{-/-}$ mice to control levels. Food intake remains unchanged. Data are presented as mean ± SEM. Open stars: treatment effect; solid stars: genotype × treatment interaction (*post-hoc*: Newman-Keuls); One star $p<0.05$, two stars $p<0.01$, three stars $p<0.001$. See also Supplementary Figure S5.

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 have a critical role in mediating reward processes (Le Merrer *et al*, 2009). Interestingly, *Oprm1*$^{-/-}$ 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$^{2.0}$ 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*$^{-/-}$ mice is most striking. Remarkably, oxytocin release in this region has been demonstrated to have 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 the cellular and molecular level, mutant mice also show most features characterizing ASDs in animal and clinical research. *Oprm1*$^{-/-}$ 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 (Supplementary Table S8) 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 the findings of ASD behavioral features elicited by increased monoaminergic activity (Bortolato and Shih, 2011). Mutant mice also showed altered expression for multiple genes

associated with 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 downregulated in striatal regions.

We next tested therapeutic utility of *Oprm1*$^{-/-}$ 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 downregulated 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 Volgel'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*$^{-/-}$ 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*$^{-/-}$ 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 lifespan as a trigger for autistic syndrome, using local or time-controlled manipulations of gene expression.

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AUTHOR CONTRIBUTIONS

JLM, JAJB, and BLK designed the experiments. JLM and JAJB performed and analyzed behavioral, immunohistochemical, qRT-PCR, and pharmacological experiments. DC performed and analyzed HPLC dosage of monoamine levels in brain tissue. CS and YS performed and analyzed electron microscopic experiments. JLM, JAJB, YS, and BLK interpreted the results. JLM, JAJB, and BLK wrote the article. JLM and BLK contributed equally to this work. All the authors discussed the results and commented on the manuscript.

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Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)