Rasd2 Modulates Prefronto-Striatal Phenotypes in Humans and ‘Schizophrenia-Like Behaviors’ in Mice

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Rasd2 is a thyroid hormone target gene, which encodes for a GTP-binding protein enriched in the striatum where, among other functions, it modulates dopaminergic neurotransmission. Here we report that human RASD2 mRNA is abundant in putamen, but it also occurs in the cerebral cortex, with a distinctive expression pattern that differs from that present in rodents. Consistent with its localization, we found that a genetic variation in RASD2 (rs6518956) affects postmortem prefrontal mRNA expression in healthy humans and is associated with phenotypes of relevance to schizophrenia, including prefrontal and striatal grey matter volume and physiology during working memory, as measured with magnetic resonance imaging. Interestingly, quantitative real-time PCR analysis indicated that RASD2 mRNA is slightly reduced in postmortem prefrontal cortex of patients with schizophrenia. In the attempt to uncover the neurobiological substrates associated with Rasd2 activity, we used knockout mice to analyze the *in vivo* influence of this G-protein on the prepulse inhibition of the startle response and psychotomimetic drug-related behavioral response. Data showed that *Rasd2* mutants display deficits in basal prepulse inhibition that, in turn, exacerbate gating disruption under psychotomimetic drug challenge. Furthermore, we documented that lack of *Rasd2* strikingly enhances the behavioral sensitivity to motor stimulation elicited by amphetamine and phencyclidine. Based on animal model data, along with the finding that RASD2 influences prefronto-striatal phenotypes in healthy humans, we suggest that genetic mutation or reduced levels of this G-protein might have a role in cerebral circuitry dysfunction underpinning exaggerated psychotomimetic drugs responses and development of specific biological phenotypes linked to schizophrenia.

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INTRODUCTION

Rasd2 (Rhes) is a GTP-binding protein (Falk *et al*, 1999) highly enriched in the striatum and nucleus accumbens of rodents and developmentally regulated by thyroid hormone activity (Vallortigara *et al*, 2008, 2009; Vargiu *et al*, 2001) and by dopamine (DA) innervation in adult rats (Harrison and LaHoste, 2006; Harrison *et al*, 2008). Rasd2 negatively

modulates G protein-coupled receptor-mediated cAMP production (Vargiu *et al*, 2004). Consistently, targeted deletion of this gene in mice results in increased striatal cAMP/PKA signaling (Errico *et al*, 2008; Ghiglieri *et al*, 2015). Besides its well-established expression within striatal GABAergic medium spiny-projecting neurons, we recently demonstrated that Rasd2 is also virtually localized in all cholinergic interneurons in humans and mice, where it affects DA D2R-dependent firing activity and signaling (Sciamanna *et al*, 2015). On the other hand, Rasd2 is able to modulate AKT and mTOR signaling cascades (Harrison *et al*, 2013; Subramaniam *et al*, 2012), well known to be involved in neurological (Lee *et al*, 2015) and psychiatric disorders (Emamian *et al*, 2004). Accordingly, the interaction between mTOR and mutant Huntingtin disrupts mTORC1 function and predisposes the striatum to the early degeneration in Huntington’s disease (Lee *et al*, 2015). On the other hand, alterations of AKT1-GSK3 β signaling in

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schizophrenic patients have identified *AKT1* as one of the potential susceptibility genes involved in such a psychiatric disorder (Emamian *et al*, 2004).

Notably, the human ortholog *RASD2* is located on the long arm of chromosome 22 (q12.3), a genomic region harboring several susceptibility loci for psychosis (DeLisi *et al*, 2002; Potash *et al*, 2003). This association is most robust in DiGeorge syndrome, which is caused by the deletion of a variable portion of this chromosome and characterized by about 30% increase in incidence of psychosis, as compared with the general population (Jonas *et al*, 2014).

Based on this background, here we tested the hypothesis that *Rasd2* may modulate in humans structural and functional prefronto-striatal systems-level phenotypes previously linked with schizophrenia. More specifically, first we evaluated the comparative pattern of fronto-striatal gene expression of *Rasd2* in the human and rodent brain. Then, we explored whether *RASD2* genetic variation is associated with prefrontal and striatal grey matter (GM) volume and activity during working memory (WM) processing in healthy subjects, as measured with structural and functional MRI. Later, we evaluated whether *RASD2* mRNA expression levels as measured by quantitative real-time PCR analysis in postmortem human brain are altered in the prefrontal cortex (PFC) of patients with schizophrenia, when compared with non-psychiatric controls. Finally, in the attempt to uncover the neurobiological substrates linked to the influences of *Rasd2*, we extended our functional studies from human to rodents. To this aim, we used mutant mice with targeted deletion of *Rasd2* (KO), and assessed their sensorimotor gating responses and motor activity, in both basal conditions and under administration of psychotomimetic drugs, such as amphetamine sulfate (Amph) and phencyclidine hydrochloride (PCP).

MATERIALS AND METHODS

In Situ Hybridization (ISH)

ISH analysis was performed according to protocols previously described (Sciamanna *et al*, 2015). Mouse fresh brain tissue was embedded in Tissue Tek (Sakura) and frozen at -80°C , and 14- μm cryostat sections were cut in the coronal plane. Human brain samples from one male (16 years, 16 h postmortem) and one female (35 years, 6 h postmortem), without history of neurological or psychiatric disorders, were dissected in the regions of dorso-lateral PFC (DLPFC) and putamen. Processing was performed as previously described (Pasqualetti *et al*, 1999). ^{35}S -labelled riboprobes for ISH were as follows: *Rasd2*, 0.5 Kb (Spano *et al*, 2004); and *RASD2*, 2.8 Kb (nucleotides 178–2971, NM_014310.3). Mouse and human hybridized sections were exposed to Biomax MR X-ray films (Kodak) for 2–4 days. Slides with human specimens were dipped in Kodak NTB emulsion for autoradiography and counter-stained with Giemsa, as previously described (Pasqualetti *et al*, 1999). Sections were examined with brightfield and darkfield light microscopy.

Human Methods

Candidate *RASD2* SNPs screening and *RASD2* mRNA expression in the postmortem human PFC. Data from 216

brains of non-psychiatric individuals, obtained from Brain-Cloud (<http://braincloud.jhmi.edu>) (Turetsky *et al*, 2007) were analyzed. Samples with age at death <0 (Vargiu *et al*, 2001) and RNA Integrity Number (RIN) <6 were not included (Supplementary Table S1). We focused on all single-nucleotide polymorphisms (SNPs, $n=66$) within *RASD2* (100 Kb upstream and downstream from the gene) available for this data set, and we explored their association with prefrontal *RASD2* mRNA expression using Bonferroni correction for the number of independent comparisons to correct for Type I errors. We selected *RASD2* intragenic SNPs—rs5750112 (UTRvariant-5'), rs6518956 (intron-variant), rs2281122 (intron-variant), rs8137058 (intron-variant), rs736211 (UTR-variant-3'), rs736210 (UTR-variant-3')—as independent comparisons, as these SNPs may alter the rate of *RASD2* mRNA expression through different mechanisms (Consortium EP, 2012). One SNP, rs6518956, survived the statistical threshold and was selected for the *in vivo* imaging analyses.

Prefrontal GM volume and prefrontal-striatal physiology in healthy humans. Healthy Caucasian volunteers from the region of Puglia, Italy were genotyped for rs6518956 (see Supplementary Tables S2 and S3) and underwent the imaging protocols. All analyses were performed in Statistical Parametric Mapping 8.0 (<http://www.fil.ion.ucl.ac.uk/spm>) with SNP effects assessed with random-effects analyses. For the evaluation of prefrontal and striatal GM volume ($n=150$), we used voxel-based morphometry (VBM) performed on 3 T structural MRI images, following standardized methods (New segmentation and Diffeomorphic registration algorithms) and using appropriate covariates as described previously (Martino *et al*, 2011) (see Supplementary Information). For WM cortical and subcortical activity ($n=160$), we analyzed 3 T BOLD fMRI prefrontal and striatal physiology during performance of the 1- and 2-Back WM task (N-Back). Statistical correction for multiple comparisons within the *a priori* defined regions of interest (ROIs) was performed as appropriate, respectively, for VBM and fMRI data (see Supplementary Materials and Methods).

Postmortem PFC samples. Samples of postmortem PFC from patients with schizophrenia and non-psychiatric controls were obtained from the Neurodegenerative Diseases Brain Bank (King's College London, UK). All tissue collection and processing was carried out under the regulations and licences of the Human Tissue Authority and in accordance with the Human Tissue Act of 2004. Clinical diagnosis of the samples was performed according to Diagnostic and Statistical Manual of Mental Disorders (DSMIII-R). Demographic characteristics of the samples are described in Table 1. Student's *t*-test analysis was performed in order to determine whether sample characteristics (age, postmortem interval (PMI) and RIN) were comparable in the two diagnostic groups. Total RNAs extraction from PFC samples and quantitative RT-PCR protocols are described in Supplementary Information.

Table 1 Neurodegenerative Diseases Brain Bank—King's College London

	NC group, <i>n</i> = 12 (mean ± SEM)	SCZ group, <i>n</i> = 9 (mean ± SEM)	df	p-Value
Age, years	61.2 ± 5.4	62.2 ± 4.4	20	<i>t</i> = −0.132
PMI (h)	36 ± 6.3	59.8 ± 7.8	19	<i>t</i> = −2.399
RIN	7 ± 0.4	6.9 ± 0.5	20	<i>t</i> = 0.086
Gender	6 M, 6 F	6 M, 4 F	$\chi^2 = 0.220$	1
Race	Caucasian	9 Caucasian, 1 Black		0.6390
Diagnosis		DSMIII-R		

Abbreviations: DSM, Diagnostic and Statistical Manual of mental disorders; F, female; M, male; NC, non-psychiatric control; PMI, postmortem interval; RIN, RNA integrity number; SCZ, schizophrenia.

Unpaired *t*-test for age, PMI, and RIN characteristics. Chi-square test for gender characteristic.

Animal Methods

Ten-to-12-week-old male and female wild-type (WT) and *Rasd2* knockout (KO) mice without PGK-neo cassette (Ghiglieri *et al*, 2015; Sciamanna *et al*, 2015), derived from mating of heterozygous animals (*Rasd2*^{+/-}), back-crossed to F11 generation to C57BL/6J strain, were used in this study. Mice were housed in groups in standard cages (29 × 17.5 × 12.5 cm³) at constant temperature (22 ± 1 °C) and maintained on a 12/12 h light/dark cycle, with food and water *ad libitum*. Experiments were performed in conformity with protocols approved by the veterinary department of the Italian Ministry of Health and in accordance to the ethical and safety rules and guidelines for the use of animals in biomedical research provided by the relevant Italian laws and European Union's directives (no. 86/609/EC). All efforts were made to minimize the animal's suffering.

Drugs. Amph, haloperidol (Hal), and PCP were obtained from Sigma (St Louis, MO). Clozapine (Clz) was obtained from RBI (Natick, MA). Amph and PCP were dissolved in saline (0.9% NaCl). Hal was dissolved in a vehicle (Veh) composed by 10% acetic acid in saline solution. The pH was brought to 6.0 with 1 M NaOH. Clz was dissolved in a Veh composed by a solution of 2% dimethyl sulfoxide (DMSO) in saline. All drugs were administered intraperitoneally (i.p.) in a volume of 10 ml/kg.

Behavior

Prepulse inhibition (PPI) of the startle reflex. Different cohorts of animals were tested at baseline or after drug injection for PPI of the acoustic startle response, measured using the SR-Lab System (San Diego Instruments, San Diego, CA, USA). The protocol has been previously described (Errico *et al*, 2008). Briefly, WT and KO naïve mice were enrolled to test baseline PPI response in males and females (see Supplementary Figure S1). Whereas another cohort of male animals was divided into the following groups: Amph (10 mg/kg) or Veh, administered 5 min before starting the test session; PCP (10 mg/kg) or Veh, administered 10 min prior to the start of the test session; Hal (3 mg/kg) or Veh, injected 30 min before starting the test session; Hal (3 mg/kg) or Veh, injected 25 min before administration of 10 mg/kg Amph or Veh, and tested 5 min later; Clz (3 mg/kg) or Veh,

injected 20 min before starting the test session; and Clz (3 mg/kg) or vehicle, injected 10 min before PCP treatment (10 mg/kg) or Veh, and tested 10 min later. The background noise level was 65 dB, and we chose to test 70, 74, and 78 dB prepulse sound levels corresponding to 5, 9, and 13 dB above background, respectively. Sound levels, expressed as dB(A) sound pressure levels, were measured as previously described (Swerdlow and Geyer, 1998). PPI was calculated as percentage (%) and used as a dependent variable with prepulse sound levels as repeated measure. Baseline PPI was analyzed by two-way ANOVA (genotype × prepulse sound levels) with prepulse sound levels as repeated measure. Effects of Amph or PCP at different doses, as well as those of Hal or Clz in preventing Amph- and PCP-induced PPI disruption, respectively, were analyzed by three-way ANOVA (genotype × treatment × prepulse sound levels) with prepulse sound levels as dependent variable. Effects of Hal or Clz alone were analyzed within genotypes by two-way ANOVA (treatment × prepulse sound levels) with prepulse as dependent variable. Startle magnitude was calculated as the average response (± SEM) to the startle trials (120 dB alone) presented during the test session.

Novelty-induced exploration. Novelty-induced exploratory response was used to assess spontaneous motor function in mice, as previously described (Errico *et al*, 2008). Locomotor activity (expressed in cm) was recorded by using a computerized video tracking system (Videotrack, Viewpoint S.A., Champagne au Mont d'Or, France). Two-way ANOVA (genotype × time) with repeated measures and Student's *t*-test were used to analyze in basal condition the time course of motor activity and the total motor activity, respectively.

Motor responses induced by drugs. Amph- and PCP-induced locomotor hyperactivity was induced according to a previous protocol (Napolitano *et al*, 2010; Usiello *et al*, 2000). Briefly, WT and KO male animals were randomly assigned to the Amph (free base 1.25, 2.5 mg/kg), PCP (3.0, 6.0 mg/kg) or specific Veh-treated groups, habituated to the experimental cage (35 × 25 × 30 cm³) for 60 min, injected with drug or Veh, and replaced in the same cage. We also analyzed WT and KO female mice for motor activity following Amph (free base 1.25 mg/kg) and PCP (3.0 mg/kg)

Figure 1 RASD2 mRNA is expressed in the putamen and in the dorso-lateral prefrontal cortex of human brain. (a–d) Radioactive ISH experiment on coronal sections of mouse brain showing *Rasd2* expression in the striatum and in the cerebral cortex. (b) High magnification image of the boxed region in panel (a) showing *Rasd2* mRNA distribution across cortical layers of the mouse brain. Autoradiographic signal appears higher in layers II/III as compared with deeper layers. (c, d) RNase-treated control section and relative high magnification showing any detectable specific signal. (e–g) Nissl staining (e) and ISH autoradiographic analysis at low (f) and high (g) magnification on sections from the postmortem human brain showing expression of RASD2 in the putamen nucleus. (h–k) Nissl staining (h, j) and ISH autoradiography (i, k) of sections from the postmortem human brain highlighting the presence of RASD2 expression in the dorso-lateral prefrontal cortex with a strong autoradiographic signal in layer V. (j, k) Nissl staining (j) and darkfield photomicrograph (k) of boxed regions in panels (h) and (i) showing the layering of human prefrontal cortex and the distribution of RASD2 mRNA, respectively. Labeled neurons appear distributed across layers II–VI, with pyramidal neurons (arrows) of layer V showing a higher density of autoradiographic silver grains (see inset in panel (k)). Cl, claustrum; Gp, globus pallidus; Pu, putamen nucleus.

or Veh administration (Supplementary Figure S2). The horizontal motor activity was evaluated during 10-min intervals over a 1-h test session through a computerized video tracking system (Videotrack, Viewpoint S.A.). Three-way ANOVA (genotype $\times$ treatment $\times$ time) with time as repeated measures was used to analyze the time course of motor activity. Two-way ANOVA (genotype $\times$ treatment) was used to analyze locomotor activity over 60-min test session. Total motor activity was analyzed by two-way ANOVA, followed by Fisher's *post-hoc* comparison.

RESULTS

Rasd2 mRNA Expression in the Human Putamen and Cerebral Cortex

Here, we first confirmed a consistent *Rasd2* mRNA expression in the mouse striatum (Errico et al, 2008; Ghiglieri et al, 2015; Vargiu et al, 2001) (Figure 1a), and extended our study of *Rasd2* gene expression pattern to the human brain, where we reported a highly enriched RASD2 mRNA expression in the putamen but not in the surrounding brain regions, such as globus pallidus and claustrum (Figure 1e–g). Moreover, consistent with previous findings in rodents (Vargiu et al, 2001), we found scattered *Rasd2* mRNA-positive neurons distributed throughout the cortical layers, with an autoradiographic signal that appeared higher in layers (L) II–III than in deeper ones (Figure 1a and b).

Conversely, RASD2 mRNA expression in humans, although detected throughout layers II–VI, showed a distinctive expression in layer V, not seen in rodents (Figure 1h–k). Interestingly, higher magnification analysis of silver grain distribution in the DLPFC showed that medium and large pyramidal-like neurons were labeled (Figure 1k, inset).

RASD2 is Associated with Prefrontal GM Volume As Well As Prefrontal and Striatal Activity During WM in Humans

We examined the association between functional variations in the RASD2 gene and a series of human imaging phenotypes related to schizophrenia. The first step was to determine which specific RASD2 genetic variants affected mRNA expression in postmortem PFC of non-psychiatric human subjects. Separate ANCOVAs on RASD2 mRNA expression as the dependent variable and RASD2 SNPs and race as predictors indicated that rs6518956 (G/A) within RASD2 is significantly associated with prefrontal RASD2 mRNA expression ($F_{(2,205)} = 5.03$, $p = 0.04$, after Bonferroni correction). More specifically, greater RASD2 mRNA expression was predicted by GG genotype (Fisher's *post-hoc* vs AG $p = 0.009$; vs AA $p = 0.003$) (Figure 2a). Age at death, gender, PMI, pH, and RIN were entered into the statistical model as covariates of no interest. We further investigated the *in vivo* association of rs6518956 with prefrontal and striatal GM volume. VBM results in healthy

Figure 2 Association of *RASD2* rs6518956 with prefrontal and striatal phenotypes in healthy humans. (a) Association of *RASD2* rs6518956 with *RASD2* mRNA expression levels in the human postmortem prefrontal cortex. Graph depicts normalized log₂ ratios (sample/reference). Data from <http://braincloud.jhmi.edu/>. Error bars indicate SE. (b) Association of *RASD2* rs6518956 with prefrontal grey matter volume in healthy Caucasian subjects. Left panel: 3-Dimensional rendering of the cluster in prefrontal cortex associated with a main effect of *RASD2* rs6518956. Image thresholded at $p < 0.005$, non-stationary cluster extend corrected. Right panel: Graph showing mean $\pm$ SE of grey matter content extracted from the cluster depicted in the left panel. (c*, c**) Association of *RASD2* rs6518956 with prefrontal and striatal BOLD response during working memory in healthy Caucasian subjects: (c*) Upper panel: 3-Dimensional rendering of the cluster in the prefrontal cortex associated with a main effect of *RASD2* rs6518956. Image thresholded at $p < 0.05$, FWE corrected. Lower panel: Graph showing mean $\pm$ SE of parameter estimated extracted from the cluster depicted in the upper panel. (c**) Upper panel: Coronal section of the interaction between *RASD2* and WM load in left putamen. Image thresholded at $p < 0.05$, FWE corrected. Lower panel: Graph showing mean $\pm$ SE of parameter estimated extracted from the cluster depicted in the upper panel.

Caucasian subjects demonstrated a main effect of rs6518956 in the left middle frontal gyrus ($x = -32, y = -0, z = 51, k = 362, Z = 3.49, p = 0.03$ non-stationary cluster-level-corrected) (Figure 2b). In particular, the GG genotype is associated with greater prefrontal GM volume compared with the other two genotype groups (Fisher's *post-hoc* vs. AG $p = 0.001$; vs AA $p = 0.003$). rs6518956 was not significantly associated with striatal (putamen/caudate) GM volume. Then we examined the association of rs6518956 with BOLD-fMRI brain activity of healthy Caucasian individuals during performance of the 1- and 2-Back WM task. There was no effect of genotype or interaction with WM load on behavioral performance (all $p > 0.2$). Thus the effect of genotype on brain responses during WM processing in this sample reflected how the brain processed WM and not how individuals scored on the task. Results of the fMRI analyses

indicated a main effect of rs6518956 on BOLD signal change in left middle frontal gyrus ($x = -34, y = 0, z = 42; k = 110; Z = 3.93; p = 0.035$ FWE-corrected) (Figure 2c*), with GG subjects showing greater prefrontal activity compared with GA and AA individuals (*post-hoc*: vs AG $p = 0.00007$; vs AA $p = 0.004$). fMRI results also demonstrated a *RASD2* by WM load interaction in the left putamen ($x = -26, y = 12, z = 10; k = 243; Z = 3.88; p = 0.041$ FWE-corrected) (Figure 2c**). More specifically, *post-hoc* analysis with *t*-test for dependent samples of BOLD response from this cluster indicated that GG and AA subjects have greater striatal activity compared with GA individuals and that this effect is more evident at 2-Back (1-Back vs 2-Back: AA, $p = 0.02$; AG, $p = 0.11$; GG, $p = 0.007$). To address whether functional results in PFC might be related to structural differences among the three *RASD2* genotype subgroups, we also performed a Pearson's

test between GM and BOLD signal change in the ROIs in a subgroup of 120 subjects (35 AA, 63 AG, 22 GG) who had both structural and functional scans. Results did not show any significant correlation between GM volume and BOLD signal change both in the entire sample of healthy subjects ($r=0.1$, $p=0.27$) and within the three different *RASD2* genotype subgroups (35 AA: $r=-0.05$, $p=0.74$; 63 AG: $r=0.06$, $p=0.59$; 22 GG: $r=-0.0009$, $p=0.99$, results not shown), suggesting that rs6518956 modulation of prefrontal activity during WM is not driven by local structural differences. Moreover, we did not find any relationship between prefrontal GM volume (in the same cluster as above) and striatal activity (extracted from the contrast showing *RASD2* rs6518956 by WM load interaction in putamen: $x=-26$, $y=12$, $z=10$; $k=243$), both in the entire sample of healthy subjects ($r=-0.07$, $p=0.41$) and within the three *RASD2* genotype subgroups (AA, $r=-0.18$, $p=0.29$; AG, $r=-0.05$, $p=0.64$; GG, $r=-0.33$, $p=0.12$, results not shown).

RASD2 mRNA Expression in the Postmortem Brains of Patients with Schizophrenia

Based on its cortical localization and considering the potential involvement of *RASD2* in prefrontal phenotypes that might be of relevance to psychiatric disorders, we then assessed whether the mRNA expression levels of this GTPase might be altered in the postmortem PFC from patients with schizophrenia. Quantitative real-time PCR analysis revealed an average 30% reduction of *RASD2* transcript in patients with schizophrenia, when compared with controls, although not statistically significant (ANCOVA with diagnosis as predictor and PMI as covariate of no interest; PFC: $F_{(1,17)}=1.429$, $p=0.2483$, Cohen's $d=0.44$; Figure 3).

Figure 3 *RASD2* mRNA expression in postmortem samples from patients with schizophrenia and non-psychiatric controls ($n=10$ SCZ, $n=12$ NC). All studies were performed by quantitative reverse transcription-PCR. Quantity means of transcript were normalized to the geometric mean of three housekeeping genes. All values are expressed as mean $\pm$ SEM.

Rasd2 Modulates Sensorimotor Gating

In order to assess the influence of *Rasd2* in modulating sensorimotor gating, known to be altered in patients and in preclinical model of schizophrenia (Braff et al, 2001), we analyzed the PPI of the acoustic startle reflex under basal conditions in *Rasd2* KO male mice ($n=15$ /genotype). Statistical analysis showed a significant PPI reduction in mutants compared with control littermates (two-way ANOVA with prepulse sound levels as repeated measures: genotype effect, $F_{(1,56)}=4.968$, $p=0.0340$; Figure 4a). Differently from what observed in males, we failed to find any PPI alteration in female KO mice (Supplementary Figure S1). Then, to investigate whether *Rasd2* deletion may affect the sensitivity of the disruptive effect induced by the DA-releaser Amph on PPI, we tested this drug at the doses of 5 ($n=9$ /genotype), 7.5 ($n=10$ /genotype), and 10 mg/kg ($n=24$ /genotype) or vehicle ($n=30$ WT, $n=32$ KO). Three-way ANOVA showed an overall significant effect of genotype ($F_{(1,280)}=10.351$, $p=0.0016$) and treatment ($F_{(3,280)}=5.441$, $p=0.0014$), but no genotype $\times$ treatment interaction ($F_{(3,280)}=0.258$, $p=0.8558$) or genotype $\times$ treatment $\times$ prepulse levels interaction ($F_{(6,280)}=0.834$, $p=0.5448$) (Figure 4b).

Further, we evaluated the involvement of *Rasd2* in regulating the severity of PPI disruption in mice treated with the NMDAR antagonist PCP, also known to promote striatal DA release (Javitt, 2007; Svensson, 2000). Here, we tested this hallucinogenic at the doses of 5 ($n=10$ /genotype) and 10 ($n=18$ WT, $n=20$ KO) mg/kg or vehicle ($n=30$ WT, $n=32$ KO). Three-way ANOVA showed a significant genotype ($F_{(1,228)}=9.339$, $p=0.0028$) and treatment ($F_{(2,228)}=8.527$, $p=0.0004$) effect, with no effect of genotype $\times$ treatment interaction ($F_{(2,228)}=0.206$, $p=0.8140$). Notably, we found a trend, although not significant, towards a genotype $\times$ treatment $\times$ prepulse interaction ($F_{(4,228)}=2.361$, $p=0.0541$; Figure 4c).

Hal and Clz Prevent Amph- and PCP-Induced PPI Deficits in *Rasd2* Knockouts

Later, we evaluated the ability of the antipsychotics Hal or Clz to prevent the prominent sensorimotor gating deficits induced in mutants by high doses of Amph or PCP, respectively. When administered at the dose of 3 mg/kg, Hal failed to induce any significant effect on PPI responses both in KO and WT mice (Hal, $n=9$ /genotype; Veh, $n=11$ /genotype), as indicated by the two-way ANOVA with prepulse sound levels as repeated measure (WT: $F_{(1,36)}=0.394$, $p=0.5381$; KO: $F_{(1,36)}=0.012$, $p=0.9123$; Figure 4d). On the other hand, pretreatment with 3 mg/kg Hal fully prevented the PPI deficits induced by Amph administration in both genotypes (Hal+Amph, $n=8$ WT, $n=9$ KO; Veh, $n=13$ WT, $n=12$ KO). Accordingly, the three-way ANOVA indicated no significant treatment effect (Veh vs Hal/Amph: $F_{(1,76)}=0.027$, $p=0.8701$) and no significant genotype $\times$ treatment interaction ($F_{(1,76)}=0.128$, $p=0.7222$; Figure 4e). Conversely, Amph at 10 mg/kg ($n=14$ WT, $n=16$ KO), as expected, triggered a significant PPI disruption in both genotypes (Veh vs Amph: $F_{(1,102)}=20.555$, $p<0.0001$).

Administration of 3 mg/kg Clz alone failed to cause any significant effect on PPI in both genotypes, compared with their respective Veh-treated group ($n=10$ /genotype

and treatment), as indicated by the two-way ANOVA with prepulse sound levels as repeated measure (WT: $F_{(1,36)} = 0.546$, $p = 0.4696$; KO: $F_{(1,36)} = 0.055$, $p = 0.8166$; Figure 4f). However, pretreatment with 3 mg/kg Clz, both in mutant and control mice, completely prevented the PPI deficits induced by PCP (Clz+PCP, $n = 10$ WT, $n = 12$ KO; PCP, $n = 10$ /genotype, Veh, $n = 9$ WT, $n = 10$ KO), as revealed by the three-way ANOVA (Veh vs Clz/PCP: $F_{(1,74)} = 1.744$, $p = 0.1947$; genotype $\times$ treatment: $F_{(1,74)} = 0.717$, $p = 0.4026$), which alone caused severe sensorimotor gating alteration (three-way ANOVA, Veh vs PCP, $F_{(1,70)} = 24.442$, $p < 0.0001$; Figure 4g).

Lack of Rasd2 Triggers Greater Sensitivity to Amph- and PCP-Induced Motor Stimulation in Mice

Here, we aimed at exploring the involvement of *Rasd2* deletion in stimulating motor effects elicited by the DA-releaser Amph and the NMDAR antagonist PCP. In this regard, we first measured the basal motor activity in mutants, compared with controls ($n = 10$ /genotype). Two-way ANOVA, with time as repeated measure, indicated that both naïve WT and KO animals display comparable locomotion and habituation profile to the novel cage (genotype effect: $F_{(1,90)} = 0.235$, $p = 0.6339$; genotype $\times$ time: $F_{(5,90)} = 0.262$, $p = 0.9325$; time effect: $F_{(5,90)} = 17.086$, $p < 0.0001$; Figure 5a). Then, we assessed the effect of Amph on motor activity at the doses of 1.25 and 2.5 mg/kg ($n = 12$ /genotype and treatment) or vehicle ($n = 14$ /genotype). Statistical analysis showed that Amph produces differential motor effects in the two genotypes along the test session, as indicated by the three-way ANOVA with time as repeated measure (genotype $\times$ treatment, $F_{(2,350)} = 7.503$, $p = 0.0011$; Figure 5b). The genotype $\times$ treatment interaction was also confirmed by total locomotor activity measured in KO and WT Amph-treated animals, when compared with their matched-vehicle groups (two-way ANOVA: genotype $\times$ treatment, $F_{(2,70)} = 7.503$, $p < 0.01$; Figure 5b'). Analysis at each dose showed that 1.25 mg/kg Amph evokes substantial motor hyperactivity in KO but not in WT animals, compared with their respective Veh-treated groups (three-way ANOVA, genotype $\times$ treatment interaction: $F_{(1,250)} = 17.713$, $p = 0.0001$; two-way ANOVA, treatment effect, WT: $F_{(1,125)} = 0.014$, $p = 0.9064$; KO: $F_{(1,125)} = 18.151$, $p = 0.0003$). On the other hand, 2.5 mg/kg Amph administration similarly enhanced horizontal motor activity in both genotypes, compared with their respective Veh-treated groups (three-way ANOVA, treatment effect: $F_{(1,240)} = 49.109$, $p < 0.0001$; genotype $\times$ treatment: $F_{(1,240)} = 1.084$, $p = 0.3030$). Then, we evaluated the influence of *Rasd2* on hyperlocomotion induced by the NMDAR antagonist PCP at the doses of 3 ($n = 7$ /genotype) and 6 ($n = 7$ /genotype) mg/kg or vehicle

($n = 12$ WT, $n = 10$ KO). Similar to Amph treatment, PCP triggered a different motor stimulation between genotypes, as indicated by the three-way ANOVA with time as repeated measure (genotype $\times$ treatment, $F_{(2,220)} = 3.957$, $p = 0.0263$) (Figure 5c). In particular, administration of a low dose of PCP (3 mg/kg) produced motor hyperactivity only in KO mice, compared with Veh-treated animals. Accordingly, our data indicated a significant genotype $\times$ treatment interaction (three-way ANOVA $F_{(1,155)} = 8.172$, $p = 0.0075$; two-way ANOVA, treatment effect, WT: $F_{(1,80)} = 0.068$, $p = 0.7980$; KO: $F_{(1,75)} = 25.517$, $p = 0.0001$). Moreover, administration of 6 mg/kg PCP increased horizontal locomotion in both genotypes, compared with the Veh-treated group, although with a higher magnitude in KOs (three-way ANOVA, treatment effect: $F_{(1,155)} = 77.308$, $p < 0.0001$; genotype $\times$ treatment interaction: $F_{(1,155)} = 5.674$, $p = 0.0235$). The differential motor effect of PCP between genotypes is also indicated by a significant difference in total distance travelled, as shown by the two-way ANOVA (genotype $\times$ treatment, $F_{(2,44)} = 3.957$, $p < 0.05$; Figure 5c'). As seen in male mutants, we also found in female KOs a greater hyperlocomotion induced either by Amph or PCP (Supplementary Figure S2).

DISCUSSION

It is documented that *Rasd2* is developmentally regulated by thyroid hormone (Manzano et al, 2003). Accordingly, hypothyroid rodents display a significant reduction of striatal *Rasd2* mRNA expression, restored by T3 administration (Vallortigara et al, 2008; Vargiu et al, 2001). Importantly, this transcriptional regulation is most likely mediated by thyroid receptor α (TR α) as, differently from what observed in TR α knockout mice, T3 supplementation induces an upregulation of *Rasd2* mRNA in hypothyroid TR β mutants (Vallortigara et al, 2008). *Rasd2* encodes for a small GTP-binding protein highly expressed in the striatum and nucleus accumbens of rodents (Errico et al, 2008; Vargiu et al, 2004), where it affects neuronal processes associated with cAMP/PKA and AKT signaling transduction cascades (Ghiglieri et al, 2015; Harrison and Lahoste, 2013). Furthermore, *Rasd2* also influences DA D1R- and D2R-related processes within striatal GABAergic projection neurons and cholinergic interneurons (Errico et al, 2008; Ghiglieri et al, 2015; Sciamanna et al, 2015). On the other hand, recent studies have suggested that *Rasd2*, through SUMO and mTOR pathway regulation, has a pivotal role in the genesis of neurological disorders involving the striatum, such as Huntington's disease (Lee et al, 2015; Subramaniam et al, 2010) and L-DOPA-induced dyskinesia (Subramaniam et al, 2012).

Figure 4 Evaluation of PPI in drug-free and psychotomimetic-treated *Rasd2* mutant mice. (a) Baseline PPI in naïve *Rasd2* mutant and control mice ($n = 15$ /genotype). (b) PPI in mice treated with 5 mg/kg ($n = 9$ /genotype), 7.5 mg/kg ($n = 10$ /genotype), and 10 mg/kg ($n = 24$ /genotype) Amph or Veh (WT, $n = 30$; KO, $n = 32$). (c) PPI values for 5 mg/kg ($n = 10$ /genotype) and 10 mg/kg (WT, $n = 18$; KO, $n = 20$) PCP or Veh (WT, $n = 30$; KO, $n = 32$) treatment. (d) Effect of 3 mg/kg Hal ($n = 9$ /genotype) on PPI in KO and WT mice. Veh-treated group ($n = 11$ /genotype) was used as control. (e) PPI response in mice treated with 3 mg/kg Hal plus 10 mg/kg Amph (WT, $n = 8$; KO, $n = 9$), 10 mg/kg Amph (WT, $n = 14$; KO, $n = 16$) or Veh (WT, $n = 13$; KO, $n = 12$). (f) Effect of 3 mg/kg Clz ($n = 10$ /genotype) on PPI in KO and WT mice. Veh-treated group ($n = 10$ /genotype) was used as control. (g) PPI response in mice treated with 3 mg/kg Clz plus 10 mg/kg PCP (WT, $n = 10$; KO, $n = 12$), 10 mg/kg PCP ($n = 10$ /genotype), or Veh (WT, $n = 9$; KO, $n = 10$). PPI was measured at three different prepulse intensities (5, 9, and 13 dB(A) above background). Percentage of the PPI was used as a dependent variable. All values are expressed as the mean $\pm$ SEM. Genotypes and treatment are as indicated.

Figure 5 Amphetamine and PCP trigger motor stimulation in mice. (a, a') Basal motor activity in naïve WT and KO mice ($n=10$ /per genotype). (b, b') Horizontal motor activity induced by administration of 1.25 mg/kg Amph ($n=12$ /genotype) and 2.5 mg/kg Amph ($n=12$ /genotype) or Veh ($n=14$ /genotype). (c, c') Locomotor activity induced by 3.0 mg/kg PCP ($n=7$ /genotype) and 6.0 mg/kg PCP ($n=7$ /genotype) or Veh (WT, $n=12$; KO, $n=10$) administration. Locomotion is expressed as distance travelled (cm), measured every 10 min over a 60-min test session (a–c) and as total motor activity (a'–c'). All values are expressed as mean $\pm$ SEM. ** $p < 0.01$, *** $p < 0.0001$ vs Veh-treated group within genotype. Genotypes and treatments are as indicated.

In the present study, we documented not only that *RASD2* mRNA is highly expressed in human putamen but also, even more interestingly, we found for the first time its expression throughout layers II–VI of PFC, with stronger levels in pyramidal neurons of layer V, not found in the rodent brain. Layer V pyramidal neurons of the DLPFC are involved in

WM processing (Goldman-Rakic, 1995), and their dysfunction has been reported in patients with schizophrenia. Although further studies are required, we cannot exclude that the prominent localization of *RASD2* mRNA in human layer V pyramidal neurons might suggest a modulatory role of this GTPase on neuronal activity within this cortical layer.

Furthermore, in line with its neuroanatomical localization, our imaging findings also provide evidence for a role of *RASD2* in modulating prefrontal and striatal phenotypes in humans. Consistently, a non-coding variation in the *RASD2* gene (rs6518956), which affects *RASD2* mRNA levels in human prefrontal postmortem tissue, also predicts *in vivo* phenotypes of prefrontal GM volume, as well as prefrontal and striatal activity during WM processing in healthy subjects. Our results also indicate that the GG genotype of rs6518956 is associated with greater prefrontal *RASD2* mRNA expression relative to the A allele, and suggest a functional role of this SNP on modulation of prefrontal *RASD2* protein levels. Consistently, the GG genotype also predicts greater prefrontal GM volume relative to the AG and AA genotypes in healthy individuals, thus leading to the hypothesis that *RASD2* may be implicated in prefrontal plasticity, likely through the modulation of key neurodevelopmental pathways, such as AKT1 and mTOR (Costa-Mattioli and Monteggia, 2013; Tan *et al*, 2008). Surprisingly, we did not find any effect of rs6518956 on striatal (putamen/caudate) GM volume. One speculation is that this SNP, which was selected on the basis of its association with prefrontal mRNA expression, affects primarily GM volume in the cortex, as also supported by results of the uncorrected whole brain exploratory analysis (Supplementary Table S4). Additional studies on postmortem striatal tissues of healthy humans might elucidate the importance of rs6518956 in modulating *RASD2* mRNA in this region.

Furthermore, in spite of similar WM performance, our fMRI results indicate that the GG genotype is associated with greater PFC activity during WM relative to AG and AA genotypes in healthy subjects, and that this effect is not driven by local structural differences. These findings suggest that GG subjects are inefficient in performing the WM task, because they need to recruit more neural resources to achieve a similar level of performance. Interestingly, *RASD2* by WM load interaction was found in putamen such that both homozygous genotypes have greater activity at 2-Back. One possible explanation is that rs6518956 effect on putamen activity only becomes manifest when greater putamen engagement is required by the more difficult task (2-Back), which might require additional neuronal resources beyond PFC. This interpretation is consistent with the results of the association in prefrontal activity, which is instead manifest at both 1- and 2-Back. Further studies are warranted to investigate how *RASD2* genetic variation interacts with diagnosis of schizophrenia in affecting imaging phenotypes related to the disorder.

Quantitative real-time PCR studies in the postmortem PFC of patients with schizophrenia indicate a mild reduction of prefrontal *RASD2* mRNA, when compared with non-psychiatric controls. However, given the small number of brain samples here analyzed, future studies are necessary to establish 'whether' and 'to which extent' changes in prefrontal *RASD2* mRNA expression occur in patients with schizophrenia.

Modeling endophenotypes of psychiatric disorders in animal models represents a preclinical approach helpful to shed light on the pathophysiology of these disorders in humans (Jones *et al*, 2011). Within this framework, the PPI of the startle reflex is one of the most frequently used cross-

species paradigm to evaluate the sensorimotor gating response, known to be disrupted in schizophrenia, and proposed as a candidate neurophysiological endophenotype of this psychiatric disorder (Gottesmann, 2003). In line with the evidence that psychotomimetic drugs, such as Amph and PCP, trigger PPI deficits in both animals and humans, administration of these drugs represents the most widely validated experimental strategy to model schizophrenia-like symptoms in rodents (Braff *et al*, 2001; Brisch *et al*, 2014; Gargiulo and Landa De Gargiulo, 2014; Jones *et al*, 2011). Based on this consideration, in the attempt to move *Rasd2* functional characterization from human to mouse, we evaluated whether lack of this GTPase in mutants influences sensorimotor gating responses in drug-free condition and after Amph or PCP treatment.

Considering the pivotal role of DA system in regulating PPI response (Braff *et al*, 2001), and coherently with an involvement of *Rasd2* in the modulation of this neurotransmission (Errico *et al*, 2008; Harrison and LaHoste, 2006; Sciamanna *et al*, 2015), our behavioral data indicate that targeted deletion of this gene induces significant sensorimotor gating deficits in naïve male mutants. Moreover, we also documented that administration of both Amph and PCP triggers prominent PPI deficits in mutants, when compared with their matched-treated controls. Nevertheless, in light of the significant basal sensorimotor gating disturbance of naïve mutants, we argue that the greater PPI alterations found in treated knockouts are most likely due to an additive effect of the drugs, rather than to a different sensitivity of the genotypes to Amph and PCP. In line with this interpretation, we failed to find in KO mice a significant left-shift in the dose-response curve of psychotomimetic drug-induced PPI deficits.

Pharmacological and genetic studies in mice have indicated a consistent role of striatal DA transmission at D2 receptor (D2R) sites in regulating sensorimotor gating responses (Mansbach *et al*, 1988; Ralph *et al*, 2001; Xu *et al*, 2002). In agreement with these findings, we argue that defective basal gating processes, along with the exacerbated magnitude of psychotomimetic drug-induced PPI deficits found in *Rasd2* mutants, might be linked to an altered striatal transmission.

Consistently, pretreatment with the DAergic D2R antagonist Hal in *Rasd2* knockouts completely prevents the dramatic PPI alterations triggered by high dose of Amph.

On the other hand, the finding that Clz prevented the PPI deficits seen in PCP-treated KO mice is consistent with the pharmacodynamic profile of Clz, which binds to D2R and blocks 5HT_{2A} receptors with high affinity, thus preventing further dopamine release within the striatum (Seeman, 2014; Vauquelin *et al*, 2012). However, as Clz is a broad-spectrum ligand (Wenthur *et al*, 2014), at present its specific mechanism of action in *Rasd2* KOs is still unclear. One possibility is that Clz could normalize DAergic-mediated signaling in these mice, thereby preventing their abnormal gating disturbances.

Intriguingly, despite normal spontaneous locomotion, we found that lack of *Rasd2* triggers a striking hypersensitivity to the Amph- and PCP-induced motor stimulation in mutants, indicating an inhibitory role exerted by this GTPase on striatal DA-mediated transmission under phasic conditions, possibly via inhibition of cAMP/PKA signaling cascade (Kelly *et al*, 2007; Pascoli *et al*, 2005; Svenningsson *et al*, 2003).

In a recent work, we reported that the lack of *Rasd2* triggers higher responsiveness to caffeine and, more intriguingly, specific gender-sensitive electrophysiological, biochemical, and behavioral phenotypes (Ghiglieri *et al*, 2015). Here, we further confirmed a relevant interaction between the gender and the appearance of peculiar sex-sensitive phenotypes in *Rasd2* mutants. Consistently, while we demonstrated that *Rasd2*-targeted deletion prominently enhanced Amphetamine (Amph) and PCP-induced motor stimulation in both genders, we found that knockout females were protected against PPI dysfunction, seen in males. Given the established role of estrogen hormones in regulating the onset and severity of symptoms in patients with schizophrenia (Kulkarni *et al*, 2012), the gender-dependent PPI responses found in *Rasd2* mutants represent an intriguing translational feature to decipher in future experiments. Accordingly, this preclinical model may provide a valuable *in vivo* tool to unveil the molecular determinants underlying the obscure interaction among sexual hormones and sensorimotor gating processes reported in humans (Kumari, 2011). Importantly, in support of previous clinical findings pointing to an association between *RASD2* and schizophrenia in a subgroup of Taiwanese patients (Liu *et al*, 2008), present data provided neuroanatomical evidence that *RASD2* mRNA is expressed in the putamen, as well as in the cortical layer V.

In addition, we demonstrated that variation in the gene coding for *RASD2* (rs6518956) affects *in vivo* prefrontal and striatal phenotypes in healthy human subjects that are relevant to schizophrenia. However, the different cortical *Rasd2* expression pattern between humans and mice represents a main concern for our cross-species studies and, in turn, could explain the absence of gross cognitive impairment in mutants (Supplementary Figure S3).

Taken together, the reported deficits in sensorimotor gating and the exaggerated behavioral responses to Amph and PCP found in KOs prompt us to suggest that *Rasd2* mutants may represent a novel genetic mouse model recapitulating the relationship between altered striatal neurotransmission and the emergence of specific subgroup of schizophrenia-like symptoms.

Finally, considering the transcriptional regulation of *Rasd2* by thyroid hormone (Vargiu *et al*, 2001), and the ability of this G-protein to act as a physiological 'brake' on striatal DA neurotransmission, we speculate that potential endocrine dysfunctions altering *Rasd2* mRNA expression could influence central DA signaling and indirectly impact on the vulnerability to develop psychiatric disorders, including schizophrenia.

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AUTHOR CONTRIBUTIONS

AU and AB designed the research and approved the final version of the paper. ADM, DV, FN, ADG, MP, VM, BP, FE, MTA, BG, LF, and PT performed the research. ADG, GB, FE, AU, MP, and AB analyzed the data. DV, FN, ADG, AB, and AU wrote the paper.

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