Transient Downregulation of DabI Protein Levels during Development Leads to Behavioral and Structural Deficits: Relevance for Psychiatric Disorders

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Psychiatric disorders have been hypothesized to originate during development, with genetic and environmental factors interacting in the etiology of disease. Therefore, developmentally regulated genes have received attention as risk modulators in psychiatric diseases. Reelin is an extracellular protein essential for neuronal migration and maturation during development, and its expression levels are reduced in psychiatric disorders. Interestingly, several perinatal insults that increase the risk of behavioral deficits alter Reelin signaling. However, it is not known whether a dysfunction in Reelin signaling during perinatal stages increases the risk of psychiatric disorders. Here we used a floxed *dabI* allele to study whether a transient decrease in DabI, a key component of the Reelin pathway, is sufficient to induce behavioral deficits related to psychiatric disorders. We found that transient DabI downregulation during perinatal stages leads to permanent abnormalities of structural layering in the neocortex and hippocampus. In contrast, conditional inactivation of the *dabI* gene in the adult brain does not result in additional layering abnormalities. Furthermore, perinatal DabI downregulation causes behavior impairments in adult mice, such as deficits in memory, maternal care, pre-pulse inhibition, and response to cocaine. Some of these deficits were also found to be present in adolescence. We also show that D-cycloserine rescues the cognitive deficits observed in floxed *dabI* mice with layering alterations in the hippocampus and neocortex. Our results indicate a causal relation between the downregulation of DabI protein levels during development and the structural and behavioral deficits associated with psychiatric diseases in the adult. *Neuropsychopharmacology* (2014) **39**, 556–568; doi:10.1038/npp.2013.226; published online 16 October 2013

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INTRODUCTION

Dysregulation of key developmental processes, caused by environmental and/or genetic risk factors, is associated with the pathogenesis of neuropsychiatric diseases (Ansorge *et al*, 2007; Brown and Patterson, 2011; Krishnan and Nestler, 2008; Nestler and Hyman, 2010). For example, maternal infection and early-life stress have been proposed as environmental risk factors for psychiatric disorders, although little is known about the mechanisms involved (Ansorge *et al*, 2007; Brown and Patterson, 2011; Krishnan and Nestler, 2008; Nestler and Hyman, 2010). Nevertheless, developmental dysregulation of a few factors, such as Disc1 (Li *et al*, 2007), NR1 in cortical interneurons (Belforte *et al*, 2009), serotonin (Ansorge *et al*, 2008; Ansorge *et al*, 2004),

and dopamine (Kellendonk *et al*, 2006), have been shown to cause dysfunctions related to these diseases.

Reelin, an extracellular protein essential for neuronal migration, has been shown to regulate glutamatergic neurotransmission in both developing and adult neurons (Pujadas *et al*, 2010; Tissir and Goffinet, 2003). Reelin signals through ApoER2 and VLDLR receptors and leads to the phosphorylation of the adapter Dab1, which subsequently activates several pathways. The phenotypes of Dab1- and ApoER2/VLDLR-knockout mice are indistinguishable from Reelin-deficient mice, thereby indicating that these signaling components are essential for the Reelin pathway (Hiesberger *et al*, 1999; Howell *et al*, 1997b; Tissir and Goffinet, 2003). Reelin expression is decreased in psychiatric disorders. In addition, *reelin* genetic variants are associated with these diseases, and Reelin overexpression protects against psychiatric disease-related phenotypes (Fatemi *et al*, 2000; Jia *et al*, 2010; Kim and Webster, 2009; Teixeira *et al*, 2011; Torrey *et al*, 2005). Interestingly, perinatal insults that cause behavioral deficits in the adult, such as alcohol exposure during pregnancy, maternal viral infection or exposure to Poly I:C, and serotonin

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dysfunction, alter the Reelin signaling pathway (Fatemi *et al*, 1999; Hashimoto-Torii *et al*, 2011; Janusonis *et al*, 2004). Thus, it has been proposed that the dysregulation of this pathway during development is crucial for psychiatric-related deficits; however, this hypothesis has not been tested.

Mice homozygous for a floxed *dab1* allele (flDab1) are hypomorphic for Dab1 protein at P1 (Pramatarova *et al*, 2008). Here we used this animal model, in which the Dab1 expression is reduced only during perinatal stages, to test whether a transient reduction of the Reelin pathway is sufficient to cause the structural and behavioral dysfunctions related to psychiatric diseases. We show that Dab1 downregulation during perinatal stages leads to permanent layering alterations and to deficits in cognition and maternal care. In addition, these mice have increased cocaine response and deficient pre-pulse inhibition (PPI) after exposure to corticosterone, thus suggesting a gene-environment interaction. Furthermore, we demonstrate that D-cycloserine rescues the cognitive deficits observed. These data suggest that the downregulation of Dab1 during development is sufficient to cause both the structural and behavioral deficits related to psychiatric diseases.

MATERIALS AND METHODS

Animals

Floxed *dab1* mice (Pramatarova *et al*, 2008) and *Ubiquitous CreER^{T2}* mice (Jackson Laboratories; B6.Cg-Tg(UBC-cre/ESR1)1Ejb/J)) were maintained on a C57BL/6J background, and floxed Dab1 mice were backcrossed for five generations in our laboratory. Mutant mice and their littermate controls were housed in groups (two to six mice per cage) and maintained in a 12 h light/dark cycle with access to food and water *ad libitum*. Experiments were conducted blind and in compliance with local animal care protocols and European guidelines. We used three genotypes: *dab1^{wt/wt}* (WT), *dab1^{flox/flox}* (flDab1), and *UbiCreER^{T2}/dab1^{flox/flox}* (Cre/flDab1). Mice were either injected with tamoxifen (TMX) (Cre/flDab1 + TMX) or not (Cre/flDab1). Guided by a pilot study, we tested Cre/flDab1 + TMX mice only when there were no differences between WT and flDab1 animals. All experiments were performed with male mice unless stated otherwise.

Tamoxifen Injection

In order to obtain temporally controlled, ubiquitous Cre-mediated recombination in Cre/flDab1 mice, we administered TMX injections (30 mg/ml in 1:10 alcohol:sunflower oil). Seven-week-old mice received daily intraperitoneal injections of 180 mg/kg for 3 days, at least 5 weeks before behavioral tests. In tests involving Cre/flDab1 animals, TMX was also injected in WT and flDab1 animals. Although we did not include Cre/flDab1 animals non-injected with TAM in all experiments, previous studies have shown that 4 weeks after TMX-treatment mice showed unaltered behaviors when compared with non-injected controls, except in the forced-swim-test (Vogt *et al*, 2008).

Histology

After perfusion with 4% paraformaldehyde in PBS ($N_{WT} = 9$, $N_{flDab1} = 7$, $N_{Cre/flDab1} = 4$, $N_{Cre/flDab1 + TMX} = 9$), brains were cryoprotected and sliced on a cryostat (50 μ m thick). Sections were stained with Nissl dye and mounted in Eukitt (Panreac). The neuronal density in layer I (V1 and S1BF areas) and the thickness of the pyramidal layer in the CA1 region were measured (three to six mice per genotype, three sections/mice, and three replicates per section). The layering and thickness of neocortical areas were also analyzed. We also evaluated the degree of hippocampal atrophy across genotypes. The subiculum, CA1, and CA3 (subdivided into dorsal and ventral) regions of each animal were classified as 'normal', 'moderate', or 'severe' phenotype on the basis of the presence of specific morphological traits, exemplified in Supplementary Figure S3. For immunohistochemistry, sections were immunoreacted with a Tbr1 antibody (1:1000; Abcam). After incubation with a secondary antibody and the ABC complex (Vectastain ABC kit; Vector Laboratories), sections were developed in a diaminobenzidine/nickel solution and mounted in Eukitt.

Western Blot

Brains from four mice per genotype were removed at several ages (E14, P1, P7, P14, and adult >P60). The procedure used was as previously described (Pujadas *et al*, 2010). Briefly, forebrains were lysed, and samples were resolved in SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Blots were incubated in primary antibody diluted in TBST-5% non-fat milk for 2 h. After incubation with a secondary antibody for 1 h, membranes were developed with the ECL+ system (GE Healthcare). The following primary antibodies were used: sheep anti-mDab1 antibody (1:1000, Exalpha Biol.) and affinity-purified antibody anti-Dab1 (B3) (1:2000; (Howell *et al*, 1997a)), mouse anti-Reelin (1:2000, Millipore), rabbit anti-ApoER2 (1:3000, ab 2561, Gift of J Herz), rabbit anti-VLDLR (1:3000, 2897, gift of J Herz), mouse anti-tubulin (1:100 000, Covance); and mouse anti-Actin (1:150 000, Chemicon).

RNA Purification and qRT-PCR Analysis

RNA from brain samples was obtained using Trizol (Invitrogen) as previously described (Pujadas *et al*, 2010). RNA quality and concentration were determined by absorbance at 260 and 280 nm in a nanodrop (Agilent) and by analysis using a bioanalyzer (Agilent). Two nanograms of RNA per sample were reverse-transcribed and amplified using the RETROscript kit (Ambion). For gene expression assays, we used FAM dye-labeled TaqMan MGB probes specific for Dab1 (Mm00438366_m1), Reelin (Mm00465200_m1), and Actb (Mm00607939_s1). Reactions were developed in a 96-well plates in an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). For quantification, we used three replicates per sample from four mice, per genotype, per age group. Differences in PCR efficiency were corrected, and data were analyzed using the Relative Expression Software Tool (REST-384) (Pfaffl *et al*, 2002).

Corticosterone Treatment

Adult mice (two to three months of age) were given corticosterone (Sigma) in their drinking water (25 mg/l) for 3 weeks, as previously described (Gourley and Taylor, 2009; Teixeira *et al*, 2011). Mice were tested 1 week after the end of treatment.

D-Cycloserine and Clozapine Injections

D-cycloserine (Sigma) was injected at a concentration of 20 mg/kg 20 min before behavioral tests. Clozapine (Sigma) was injected at a concentration of 0.63 mg/kg 30 min before behavioral tests.

Rota-Rod

On day one, mice were habituated to walking on the rota-rod for 1 min (Panlab) at 4 r.p.m. In the following 3 days, they were given two trials daily with the rotor-rod accelerating from 4 to 40 r.p.m. over 5 min. The average latency to fall was calculated for each mouse ($N_{WT} = 6$, $N_{flDab1} = 8$, $N_{Cre/flDab1 + TMX} = 7$).

Social Recognition

The social recognition test was performed as described (Silverman *et al*, 2010). Briefly, mice were allowed to explore an arena with three sections for 5 min. Next, a mouse inside a wire container was placed on one side of the arena and an empty wire container in the other section. Mice were allowed to explore these containers for 10 min. After this time, a novel mouse was placed in the empty container. The time the mice spent inspecting each of the containers was recorded for 10 min ($N_{WT} = 6$, $N_{flDab1} = 8$, $N_{Cre/flDab1 + TMX} = 7$).

Pup Retrieval

Poor maternal care is often seen in schizophrenic patients (Bosanac *et al*, 2003; Riordan *et al*, 1999; Wan *et al*, 2007). To assess maternal care, we used the 'pup retrieval' test. In this test, the litter was removed from the nest at P7. Next, three pups were placed at the opposite side of the cage, and the time the mother took to retrieve all three pups and carry them back to the nest was measured. There was no significant difference between the number of pups in the litters of WT (6.9 ± 0.4) and *flDab1* (6.4 ± 0.5) mothers. We performed this test with the pups that shared the same genotype as the mothers and all the pups heterozygous for the floxed *Dab1* transgene for both genotypes of mothers ($N_{WT/WT} = 9$, $N_{flDab1/flDab1} = 6$, $N_{WT/Het} = 12$, and $N_{flDab1/Het} = 17$; represented as mother's genotype/pup's genotype).

Open Field (Anxiety)

The open field (OF) apparatus consisted of an open white arena measuring 43×43 cm. Mice were allowed to walk freely over a 20-min trial. The time the mice spent in the center vs the time spent at the periphery of the arena (center: area 6 cm away from the walls), as well as the total distance traveled, were recorded ($N_{WT} = 13$, $N_{flDab1} = 12$, $N_{Cre/flDab1 + TMX} = 8$, and $N_{Cre/flDab1} = 15$).

Cocaine Sensitization

Mice (Habituation: $N_{WT} = 5$, $N_{flDab1} = 5$, $N_{Cre/flDab1 + TMX} = 5$, $N_{Cre/flDab1} = 5$; Cocaine: $N_{WT} = 10$; $N_{flDab1} = 9$; Clozapine and D-cycloserine experiment: $N_{WT} = 20$, $N_{flDab1} = 18$, $N_{flDab1 + Cyc} = 9$, $N_{flDab1 + Clz} = 17$; and Cocaine adolescents: $N_{WT} = 7$ F + 3M, $N_{flDab1} = 7$ F + 3M) were habituated to the OF box for 2 days. Starting the following day, mice were either injected intraperitoneally with cocaine (7.5 mg/kg) or saline (Habituation group), and immediately placed in the OF box; this procedure was repeated for 5 consecutive days. Locomotion was recorded for 20 min using the Smart Junior software (Panlab).

Pre-Pulse Inhibition

The PPI apparatus and procedure were as previously described (Teixeira *et al*, 2011). Mice were habituated to the apparatus with a background noise of 62 dB and then exposed to six blocks of eight trial types in pseudo-random order. The eight trial types were as follows: trial 1 (startle only), 40 ms of 120 dB background noise, 8000 Hz sound; trials 2–7 (pre-pulse trials), 40 ms of 120 dB, 8000 Hz sound preceded for 140 ms by a 20-ms pre-pulse sound of 70, 74, 78, 82, 86, or 90 dB; and trial 8, 62 dB (Untreated: $N_{WT} = 16$, $N_{flDab1} = 21$, $N_{Cre/flDab1 + TMX} = 9$. Corticosterone: $N_{WT} = 14$, $N_{flDab1} = 11$, $N_{Cre/flDab1 + TMX} = 12$).

Water Maze

The apparatus and procedure for training was as previously described (Teixeira *et al*, 2006). *Hidden version*: guided by extra-maze visual cues, mice were trained to find a submerged platform placed in a fixed location of the pool. On each of the 6 (adults) or 4 (adolescent) training days, mice received six training trials (presented in two blocks of three trials). The order of the start locations was pseudo-randomly varied throughout training. The trial was complete once the mouse found the platform or when 60 s had elapsed. When the mouse failed to find the platform in a given trial, it was guided onto the platform. During the probe test, the platform was removed, and the search pattern of the mouse was recorded for 60 s. Adults D-cycloserine experiment: $N_{WT} = 5$, $N_{flDab1} = 8$, $N_{flDab1 + Cyc} = 6$; Adults clozapine experiment: $N_{flDab1} = 9$, $N_{flDab1 + Clz} = 8$; Adolescents experiment: $N_{WT} = 7$ F + 3M, $N_{flDab1} = 9$ F + 3M.

Cued Water Maze

In this version of the water maze, mice were trained to find a platform marked with a local cue. Mice were given three trials a day for 4 days. Latency to find the platform was recorded ($N_{WT} = 8$, $N_{flDab1} = 14$).

Statistical Analysis

Statistical differences were established using a two-tailed Student's *t*-test or ANOVA followed by an LSD or Bonferroni *post hoc* test. Data are expressed as mean $\pm$ SEM.

RESULTS

Transient Downregulation of Dab1 Protein in flDab1 Mice

Mice homozygous for a flDab1 have been shown to be hypomorphic for Dab1 protein at P1. To analyze the time course of Dab1 expression in these mutants, we quantified Dab1 protein levels using western blots at several developmental stages (E14, P1, P7, P14, and adult; four mice/genotype/time point). As shown in Figure 1a (and Supplementary Figure S1), flDab1 mice displayed lower levels of Dab1 than WT mice at P1 ($t_{(6)} = 4.24$, $p = 0.005$) and P7 ($t_{(6)} = 2.92$, $p = 0.03$) but expressed Dab1 at WT levels at E14 ($t_{(6)} = 0.12$, NS), P14 ($t_{(6)} = 1.19$, NS), and in the adult ($t_{(6)} = -1.72$, NS). These results indicate that flDab1 mice experience a transient decrease in Dab1 protein during perinatal stages. Consistent with previous studies, Dab1 levels remained high in the adult brain.

To understand whether the Dab1 downregulation observed in flDab1 mice was due to differences at the RNA or protein level, we performed RT-PCR for *dab1* at P1 and adult stages in WT and flDab1 animals (four mice/genotype/time point) and analyzed the data using the REST software tool (Pfaffl et al, 2002). Compared with WT animals, flDab1 mice showed lower *dab1* expression by a factor of ~ 6 at both P1 and adult stages (REST©: $p < 0.05$), thereby indicating that flDab1 mice express lower Dab1 mRNA at both ages (see Discussion).

To test whether a further disruption of Dab1 in adulthood leads to deficits distinct from those in flDab1 animals (see below), we generated a conditional *dab1* knockout mouse line by crossing flDab1 mice with a ubiquitous Cre line (*UbiCreER^{T2}*) in which TMX-induced recombination leads to the inactivation of *dab1* and the expression of beta-galactosidase (Cre/flDab1 + TMX; Figure 1b). In these mice, Dab1 expression was dramatically reduced (Figure 1a), whereas non-injected mice (Cre/flDab1) expressed Dab1 at levels comparable to those of flDab1 animals (Supplementary Figure S2).

To study whether other components of the Reelin signaling cascade were also altered in flDab1 mice, we analyzed the expression of Reelin and the VLDLR and ApoER2 receptors. We found no differences in the expression of these proteins between genotypes at P1 (Figures 1e and f; ApoER2 $t_{(6)} = 0.114$, NS; VLDLR $t_{(6)} = 0.4146$, NS). Our western blots also confirmed that, although Reelin levels were higher at P1 than in adulthood in both genotypes (Figure 1d, $F_{(1,12)} = 127.04$, $p = 0.000$), the opposite was true for Dab1 levels (Figure 1c; $F_{(1,8)} = 26.32$, $p = 0.000$). We thus conclude that flDab1 mice display a transient and specific reduction in Dab1 protein levels during perinatal stages.

flDab1 Mice Show Permanent Structural Abnormalities that are not Affected by Adult Inactivation of the *dab1* Gene

Next, we addressed whether transient Dab1 downregulation leads to structural deficits. Although adult flDab1 mice displayed a normal gross anatomy, Nissl-staining revealed ectopic pyramidal cells in the CA1 and CA3 regions of the hippocampus (Figures 2a and b). Nissl-staining and

Tbr1-immunolabeling showed no major alterations in the lamination of the neocortex. However, flDab1 mice showed a mild disorganization of layer II, which was particularly evident in the cingulate and retrosplenial cortices (Figures 2d, e, g and h) as well as in the entorhinal cortex (Figures 4a and b) and piriform cortex (data not shown). Moreover, layer I in flDab1 mice appeared not to be as well-delineated as in WT littermate controls (Figures 2g and h; Figure 3e, f, i and j).

To determine whether such structural alterations were increased by the inactivation of the *Dab1* gene in the adult, 8-week-old Cre/flDab1 mice were injected with TMX and their cortical cytoarchitectonics was examined 2 and 12 months later. The phenotype of these mice, regarding neocortical and hippocampal layering, appeared to be indistinguishable to that of flDab1 animals (Figures 2c, f and i; Figures 3c, d, g, h, k and l; Figure 4c).

To further support these observations, we performed a quantitative analysis in the CA1 region and in the primary somatosensory (S1BF) and visual (V1) cortices (Figures 3m–o). The CA1 layering phenotype was similar in flDab1, Cre/flDab1, and Cre/flDab1 + TMX mice ($F_{(3,15)} = 36.87$, $p < 0.0001$, *post hoc* $p < 0.001$). We also confirmed the presence of ectopic neurons in neocortical layer I in all three mutant genotypes, compared with control WT littermates, in either S1BF ($F_{(3,15)} = 16.83$, $p < 0.0001$, *post hoc* $p < 0.01$) or V1 ($F_{(3,13)} = 3.587$, $p = 0.0437$, *post hoc* $p < 0.05$). The overall layering of cortical areas S1BF and V1 was similar in all four genotypes (Figures 3p, q; S1BF Group: $F_{(3,75)} = 0.16$, NS; V1 Group: $F_{(3,65)} = 0.40$, NS).

We also performed a semiquantitative comparison of the structural deficits present at the ventral and dorsal hippocampal levels. We found that the abnormal layering phenotype was more conspicuous in the ventral than in the dorsal hippocampus (Supplementary Figure S3). Finally, we were unable to find structural and layering alterations in the olfactory bulb, adult subventricular zone, or Islands of Calleja in flDab1 mice (Figures 4d–l). This observation is consistent with fact that these regions arise mainly during postnatal and adult stages.

Taken together, the present findings indicate that a transient downregulation of Dab1 protein during perinatal stages is sufficient to lead to permanent structural and layering alterations in the neocortex and hippocampus. Furthermore, we conclude that long-term inactivation of the *dab1* gene in adult stages does not further influence neuronal layering.

flDab1 Mice Present Behavioral Phenotypes Related to Psychiatric Diseases

In order to understand the behavioral outcome of perinatal disruption of Dab1, we tested WT and flDab1 mice in an extensive array of tests to elucidate the positive and negative symptoms of schizophrenia as well as cognitive deficits. Guided by a pilot study, we included TMX-injected adult *UbiCreER^{T2}/Dab1^{fllox/flox}* mice (Cre/flDab1 + TMX) in the tests, in which we did not observe a difference between WT and flDab1 mice. First, in order to rule out possible motor deficits, the three groups of mice were tested on a rota-rod. No differences in performance were observed between groups (Figures 5a and b; RPM: Day $\times$ Group: $F_{(4,36)} = 0.41$,

Figure 1 Floxed *dab1* allele (flDab1) mice exhibit decreased Dab1 protein expression. (a) *Top*: quantification of Dab1 protein levels in flDab1 animals at several time points as a percentage of WT expression levels. Dab1 is significantly reduced at P1 and P7 when compared with WT animals. *Bottom*: western blot showing Dab1 levels in WT and flDab1 animals at various developmental time points. Note that TMX administration to UbiCreER^{T2}/Dab1^{loxP/loxP} mice (Cre/flDab1) results in the loss of Dab1 protein. (b) Adult *dab1* knockout mice were obtained by crossing a ubiquitous Cre line inducible by TMX with flDab1 mice. After TMX injection, Dab1 is excised and β-galactosidase is expressed (blue staining). Arrow points to ectopic cells found in the CA1 region of all *dab1* mutants. (c) *Top*: western blot for Dab1 protein. *Bottom*: quantification revealed significantly higher levels of Dab1 in adult vs P1 mice. (d) *Top*: western blots for Reelin protein. Arrowheads show two Reelin bands, at 180 and 400 kD. *Bottom*: quantification of Reelin protein levels at P1 and adult in WT and flDab1 mice. Reelin levels were higher at P1 than in the adult, but equal between genotypes at each age group. (e) *Top*: western blot for ApoE2R. *Bottom*: quantification of ApoE2R protein levels at P1 between WT and flDab1 mice shows no difference between genotypes. (f) *Top*: western blot for VLDLR. *Bottom*: quantification of VLDLR protein levels at P1 between WT and flDab1 mice shows no difference between genotypes. Data are presented as mean ± SEM; **p* < 0.05.

NS; Group: $F_{(2,18)} = 1.64$, NS; Latency to fall: Day × Group: $F_{(4,36)} = 0.39$, NS; and Group: $F_{(2,18)} = 1.68$, NS).

The negative symptoms of schizophrenia, including lack of motivation, social withdrawal, and diminished affective responsiveness, contribute to poor functional outcomes. In order to assess social behavior, we performed the social recognition test. Mice with problems in social recognition will spend less time exploring a 'new mouse' vs a mouse

they had encountered before, whereas a general lack of social interest would reflect on a lack of preference for a container with a mouse inside vs an empty one. In this test, mice were first exposed to a three-chamber arena containing two empty wire cups. On the following day, the mice were again placed in this arena but a new mouse (Mouse 1) was placed inside one of the cups. All three groups spent significantly more time exploring the cup containing Mouse 1

Figure 2 Histological characterization of floxed *dab1* allele (*flDab1*) mice. (a–c) In Nissl-stained hippocampal (HPC) slices of *flDab1* and *Cre/flDab1* animals, ectopic pyramidal neurons are observed near the pyramidal layer in the CA1 field (arrowheads b, c). (d–i) In Tbr-1-stained sections, we observe disorganized layers I and II in the posterior cingulate cortex (PCC) in *flDab1* and *Cre/flDab1* mice. (g–i) Higher magnifications of the cingulate cortex showing disorganized layer II and an altered layer I (arrowheads h, i) in *flDab1* and *Cre/flDab1* + TMX mice. Scale bar = 250 μ m; in (a) refers to (a–c), in (d) refers to (d–f), in (g) refers to (g–i).

than the empty container (Figure 5c; Cup: $F_{(1,17)} = 26.21$, $p = 0.000$; *post hoc* $p = 0.000$). On the third day, the mice were returned to the arena, but this time a new mouse (Mouse 2) was placed in the other wire cup. No differences in exploration behavior between genotypes were detected, with all three groups preferring the new mouse *vs* the one to which they were previously exposed (Figure 5d; Novelty: $F_{(1,18)} = 22.15$, $p = 0.000$; *post hoc* $p = 0.000$).

To evaluate maternal care, we measured the time WT and *flDab1* mothers took to retrieve three pups of their litter (Figure 5e). First, we tested mothers with the same genotype as their litters; interestingly, *flDab1* mothers took more time to retrieve and place their offspring back in the nest than WT animals (Figure 5e left; $t_{(13)} = -2.38$, $p = 0.03$). To rule out the possibility that the longer latency to retrieve the *flDab1* babies was due to differences in pup behavior, we tested the mothers with heterozygous pups (Figure 5e right; $t_{(27)} = -2.33$, $p = 0.03$). Moreover, *flDab1* mothers took longer to retrieve their litter than WT mothers. This finding suggests that *flDab1* mothers are slower at retrieving pups, regardless of pup genotype and potential behavioral differences.

To further study the implication of *Dab1* downregulation in anxiousness, we performed the OFT. Although WT and *flDab1* animals spent a similar percentage of time in the center of the arena, suggesting similar levels of anxiety-like behavior, *Cre/flDab1* + TMX animals showed increased

anxiety-like behavior, as indicated by less time spent in the center of the OFT arena (Figure 5f). In contrast, *Cre/flDab1* animals that were not injected with TMX behaved in a similar manner to WT or *flDab1* mice, ($F_{(3,44)} = 3.29$, $p = 0.03$, *post hoc* $p = 0.05$).

The novelty-induced hyperlocomotion test is often used to model positive symptoms of schizophrenia (van den Buuse, 2010). *flDab1* and WT mice were placed in a novel OF environment for 20 min a day for 7 days. We observed no difference between these genotypes in the distance traveled in the OF over the 7 days, suggesting no difference in novelty-induced locomotion. However, *Cre/flDab1* + TMX maintained higher levels of locomotion during the 7 days of testing than WT and *flDab1* mice. These higher locomotion levels were not observed in non-injected *Cre/flDab1* animals (Figures 6a and b; Group: $F_{(3,14)} = 3.87$, $p = 0.03$, *post hoc* $p = 0.03$).

Positive symptoms of schizophrenia and manic behavior are also revealed by increased activity in response to psychostimulants (Geyer and Markou, 1995; Nestler and Hyman, 2010; Teixeira *et al*, 2011; van den Buuse, 2010). WT and *flDab1* showed similar levels of locomotion at baseline (Figures 6a and b); however, in response to cocaine administration, the latter displayed significantly higher locomotion than WT mice (Figures 6c and d; Group_(cocaine days): $F_{(1,17)} = 5.33$, $p = 0.03$, *post hoc* $p = 0.03$).

Figure 3 Histological characterization of the CA1 hippocampal region and SIBF and V1 neocortical areas in floxed *dab1* allele (flDab1) mice. (a–l) Representative photographs of Nissl-stained sections showing the cytoarchitectonic characteristics observed in different genotypes in the CA1 region and in neocortical areas. (m) Histograms showing average thickness of the ventral CA1 pyramidal cell layer. (n–o) Densities of ectopic neurons in layer I of SIBF and V1 regions. (p–q) Histograms showing average thickness of cortical layers in SIBF and V1 areas. or, stratum oriens; CA1, pyramidal layer; rad, stratum radiatum; slm, stratum lacunosum-moleculare; ml, molecular layers; DG, dentate gyrus; SIBF, primary somatosensory cortex, barrel field; V1, primary visual cortex; I–VI, cortical layers I–VI. Data are presented as mean $\pm$ SEM; * p < 0.05; ** p < 0.01; *** p < 0.001; Bonferroni *post hoc* test. Scale bar = 250 μ m, pertains to all panels.

Figure 4 Histological characterization of the entorhinal cortex, lateral subventricular zone, olfactory bulb and Islands of Calleja in floxed *dab1* allele (*fDab1*) mice. (a–l) Nissl-stained sections showing the cytoarchitectonics of the LEnt, OB, SVZ, and ICj regions. In the LEnt (a–c) layer II appears to be disrupted (arrowheads). Adult neurogenesis-related zones such as the OB (d–f), SVZ (g–i, arrows), and ICj (j–l, asterisks) present normal histological and layering patterns. *LEnt*, lateral entorhinal cortex; *II-III-LD-IV*, cortical layers II, III, lamina dissecans, IV; *OB*, olfactory bulb; *GCL*, granule cell layer; *IPL*, internal plexiform layer; *Mi*, mitral cell layer; *EPL*, external plexiform layer; *GL*, glomerular layer; *SVZ*, subventricular zone; *LV*, lateral ventricle; *cc*, corpus callosum; *CPu*, caudate putamen; *ICj*, island of Calleja. Scale bars = 250 μ m; scale bar in (a) refers to (a–c), in (d) refers to (d–f), in (g) refers to (g–i), and in j refers to (j–l).

***fDab1* Mice Present PPI Deficits after Corticosterone Treatment**

PPI deficits are common in patients with schizophrenia and are considered an endophenotype of the disorder. Under baseline conditions, we found no differences between WT, *fDab1*, and *Cre/fDab1* + TMX animals (Figures 6e and f; Group $\times$ Pre-pulse: $F_{(10,215)} = 1.26$, NS; Group: $F_{(2,43)} = 0.84$, NS; and Pre-pulse: $F_{(5,215)} = 13.60$, $p = 0.000$), although we detected a lower startle response in WT mice

(Supplementary Figure S4). The ‘two-hit’ model of psychiatric disease suggests that genetic alterations interact with environmental factors in the development of the disorder. One of the most salient environmental factors triggering psychiatric diseases is chronic stress (Krishnan and Nestler, 2008; Nestler and Hyman, 2010). To model chronic stress in mice, we administered corticosterone over an extended period of time. Interestingly, corticosterone-treated *fDab1* as well as *Cre/fDab1* + TMX mice presented reduced PPI as compared with corticosterone-treated WT mice (Figures 6g

Figure 5 Behavioral phenotypes related to negative symptoms of schizophrenia. (a and b) No differences in motor coordination between groups, as measured in the rotor-rod test. (c and d) In the first stage of the social recognition test, all genotypes showed preference for the container with a mouse vs the empty one, similarly there were no differences in social recognition between the groups tested, with all groups spending more time exploring the novel mouse compared with the one previously presented. (e) Floxed *dab1* allele (*flDab1*) animals took longer to retrieve and place three P7 pups back in the nest than wild-type mothers for pups of the same genotype as the mothers and for floxed *Dab1* heterozygous pups. x axis represents genotype of mother/genotype of the pups, *Het*: floxed *Dab1* heterozygous mice. (f) *Cre/flDab1* + TMX animals showed a greater tendency to avoid the center area in the OFT. Data are presented as mean $\pm$ SEM; * $p < 0.05$.

and h; Group: $F_{(2,34)} = 7.16$, $p = 0.003$, *post hoc* $p < 0.01$), with no differences in startle amplitude (Supplementary Figure S4). This finding points to a gene–environment interaction for the disruption of this behavior.

flDab1 Mice Display Cognitive Deficits that are Rescued by D-Cycloserine

Because cognitive deficits are common in schizophrenia and other psychiatric disorders (Arguello and Gogos, 2010), we tested whether memory was affected. Although most studies in mice have focused on working memory, hippocampal-dependent episodic memory is also affected in schizophrenic patients (Boyer *et al*, 2007). Given the striking hippocampal morphological phenotype, we chose to model episodic memory using the Morris Water Maze task. First, to rule out possible interference in learning by non-mnemonic deficits, we tested the mice in the cued version of the water maze. No differences were detected between genotypes in this task (Figure 7a, Day $\times$ Group:

$F_{(3,60)} = 0.29$, NS; Group: $F_{(1,20)} = 0.22$, NS; and Days: $F_{(3,60)} = 19.54$, $p = 0.000$).

Next, we tested spatial memory in the water maze using a hidden platform in a fixed location of the pool. We trained mice for 6 days in the water maze and performed a probe test on day 7. *flDab1* mice showed severe spatial memory deficits when compared with littermate controls, as indicated by the lower time spent in the target quadrant (where the platform was previously located) during the probe test (Figure 7b). D-cycloserine, a partial agonist of the NMDA receptor, has been proposed as a potential therapeutic option in schizophrenia-associated memory deficits. As Reelin controls glutamatergic neurotransmission, we tested the capacity of D-cycloserine to rescue cognitive deficits in *flDab1* mice. As shown in Figure 7b, D-cycloserine reverted the memory deficits observed in *flDab1* mutant mice (Group $\times$ Quadrant: $F_{(6,54)} = 3.54$, $p = 0.005$, *post hoc* $p < 0.001$). Next, we tested the capacity of clozapine, an atypical antipsychotic drug, to improve memory in *flDab1* mice. Clozapine, unlike D-cycloserine, was unable to enhance memory of these mice in the water

Figure 6 Behavioral phenotypes related to positive symptoms of schizophrenia and pre-pulse inhibition (PPI) deficits. (a and b) Cre/floxed *dab1* allele (flDab1) + TMX maintained higher levels of locomotion than WT and flDab1 mice over the 7 days of testing in the OFT. (c and d) flDab1 animals showed greater locomotion during the 5 days of cocaine administration than WT. (e and f) No differences in PPI were found under basal conditions between groups. (g and h) PPI was significantly reduced in flDab1 and Cre/flDab1 + TMX animals after chronic corticosterone treatment. Data are presented as mean $\pm$ SEM; * p < 0.05.

Figure 7 Cognitive deficits in floxed *dab1* allele (flDab1) mice are rescued by D-cycloserine. (a) There were no differences between genotypes in the latency to reach platform in the cued version of the water maze. (b) Performance in the spatial-reference water maze test was impaired in flDab1 animals and was rescued by D-cycloserine. flDab1 mice spent significantly less time in the target quadrant than WT and flDab1 mice treated with D-cycloserine during the probe trial at day 7. flDab1 + Cyc: flDab1 mice injected with D-cycloserine 20 min before a behavioral test. (c) Clozapine administration to flDab1 mice (flDab1 + Clz) did not improve performance in the water maze. (d and e) Clozapine administration at a dose that did not reduce baseline locomotion reduced cocaine-induced hyperactivity in flDab1 mice to WT levels. However, D-cycloserine injection had no effect on locomotion in flDab1 mice injected with cocaine. Data are presented as mean $\pm$ SEM; * p < 0.05.

maze (Figure 7c, Quadrant $\times$ Group: $F_{(3,45)} = 1.4$, NS), with both groups spending similar amounts of time (25%) in the target quadrant area. However, in flDab1 mice, clozapine (but not D-cycloserine) reduced cocaine-induced hyperlo-

comotion to levels comparable to those of cocaine-treated WT animals, at a dose that did not affect baseline locomotion (Figures 7d and e; $F_{(3,59)} = 4.59$, $p = 0.006$, *post hoc* $p < 0.05$).

Figure 8 Cognitive deficits and exacerbated cocaine-induced locomotion are already present in floxed *dab1* allele (flDab1) mice during the adolescent period. (a) flDab1 mice display memory impairments in the water maze during adolescence. (b and c) Locomotion during the 5 days of cocaine administration was higher in flDab1 adolescent animals than in WT mice. Data are presented as mean $\pm$ SEM; * $p < 0.05$.

Presence of Behavioral Deficits in Adolescent flDab1 Mice

We next tested whether the behavioral deficits observed in flDab1 mice were already present during adolescence. Twenty-one-day-old WT and flDab1 mice were trained in the water maze for 6 days. During the probe trial (Figure 8a) WT mice spent significantly more time in the target quadrant than flDab1 mice (Quadrant $\times$ Group $\times$ Sex: $F_{(3,69)} = 0.5$, NS; Quadrant $\times$ Group: $F_{(3,69)} = 5.14$, $p = 0.003$, *post hoc* $p < 0.05$).

A separate group of 21-day-old WT and flDab1 mice underwent the cocaine sensitization protocol. As with adult injected animals, flDab1 mice injected with cocaine presented significantly higher locomotion than WT-injected mice (Figure 8b and c; Group_(cocaine days): $F_{(1,16)} = 5.53$, $p = 0.032$, *post hoc* $p = 0.032$). These data suggest that the behavioral dysfunctions observed in flDab1 mice are already present during the adolescence period.

DISCUSSION

Our results demonstrate that the downregulation of Dab1 protein levels during perinatal stages has long-lasting structural and behavioral consequences. flDab1 mice, which exhibit transient downregulation of Dab1 at P1–P7, showed deficits in the water maze, maternal behavior, response to psychostimulants, and PPI after exposure to corticosterone. Moreover, although perinatal disruption of Dab1 signaling did not affect habituation to a new environment and anxiety-like behavior in the OFT, further Dab1 inactivation during adulthood did. In addition, we show that the cognitive deficits in flDab1 mice can be rescued by D-cycloserine but not by clozapine. Finally, we show that some of the behavioral dysfunctions seen in the adult are already present during adolescence. Collectively, our results indicate that a reduction in Dab1 protein levels during development is sufficient to lead to the structural and behavioral deficits related to psychiatric diseases in the adult.

In the flDab1 hypomorphic model (Pramatarova *et al*, 2008), we observed decreased Dab1 expression during perinatal stages. This decrease was transient (P1–P7 stages), with adult flDab1 mice displaying similar Dab1 protein levels to WT littermates. No expression changes were found in other components of the Reelin cascade, including Reelin and its receptors. This observation allowed us to study how a transient reduction of the Reelin signaling pathway during perinatal stages affects adult behavior and brain structure.

Regarding the mechanism of Dab1 downregulation during perinatal stages, it is well established that Reelin signaling

through the adapter Dab1 leads to its tyrosine phosphorylation and subsequent ubiquitin-dependent degradation (Feng *et al*, 2007; Kerjan and Gleeson, 2007). Consistent with such Reelin-dependent Dab1 degradation, Dab1 protein levels are higher in *reeler* Reelin-deficient mice and lower in Reelin overexpressing mice than in wild-type mice (Feng *et al*, 2007; Kerjan and Gleeson, 2007; Pujadas *et al*, 2010). It is also well established that Reelin levels are higher during development and decrease dramatically in the adult brain (Howell *et al*, 1997a). We thus propose that, although *dab1* transcription in the adult flDab1 mouse is sufficient to maintain normal Dab1 levels as a result of the low level of Reelin signaling and consequent Dab1 degradation, at P1 in flDab1 mice, *dab1* transcription is insufficient to keep Dab1 protein levels high because of the strong activation of the Reelin cascade and rapid degradation of Dab1 protein (Pramatarova *et al*, 2008). Finally, our observation that inactivation of the *dab1* gene in adult mice (Cre/flDab1 + TMX line) does not lead to further layering abnormalities is consistent with the notion that the Reelin pathways is not required to maintain the cytoarchitectonics of the adult brain after the migratory period has been completed.

Environmental factors such as maternal infection and early-life stress are thought to contribute to the development of schizophrenia and depression (Brown and Patterson, 2011; Nestler and Hyman, 2010). Interestingly, alcohol exposure *in utero*, serotonin dysfunction, and maternal infection have been shown to disrupt psychiatric disorder-related behaviors, in addition to reducing Reelin signaling (Fatemi *et al*, 1999; Hashimoto-Torii *et al*, 2011; Janusonis *et al*, 2004). For example, maternal influenza infection reduces the number of Reelin-expressing cells in the offspring (Fatemi *et al*, 1999), and Poly I:C administration during pregnancy leads to reduced Reelin levels and to behavioral phenotypes related to psychiatric disorders (Meyer *et al*, 2008). In addition, several studies have reported an association between Reelin polymorphisms and psychiatric disorders, and Reelin levels have been shown to be reduced by 50% in these patients (Fatemi *et al*, 2000; Jia *et al*, 2010; Kim and Webster, 2009; Torrey *et al*, 2005), thus further supporting the link between Reelin signaling and disease. Consistent with this, we found that Dab1 downregulation during the perinatal period leads to phenotypes related to psychiatric diseases that are exacerbated by factors associated with increased risk, such as stress and psychostimulants.

In addition to behavioral deficits, we observed cellular heterotopias in the cortex, which have also been described in the brains of subjects affected by psychiatric diseases,

including schizophrenia and bipolar disorder (Connor *et al*, 2009). Interestingly, a recent report has shown that Disc1, a schizophrenia candidate protein, acts downstream of Dab1 to regulate cortical migration (Young-Pearse *et al*, 2010). Genetic studies have implicated the *disc1* gene in the development of psychiatric disorders (Jia *et al*, 2010; Kim and Webster, 2009). Moreover, disruption of Disc1 function at P7 results in working memory deficits and reduced sociability, among other schizophrenia-related phenotypes (Li *et al*, 2007). Altogether, these data point to the convergence of the Dab1 and Disc1 pathways for the wiring of the brain during development and thus for correct adult brain function. Interestingly, although both Disc1 and NMDA dysfunction have been implicated in the pathophysiology of schizophrenia independently, a recent report suggests that these two pathways are related (Ma *et al*, 2013), with Disc1 deficiency leading to a decrease in D-serine (an endogenous agonist of the NMDA receptor) and subsequent behavioral dysfunction. This outcome may suggest a possible therapeutic function for D-cycloserine where the Dab1/Disc1 pathway may be disrupted, which is worthy of further investigation.

The Reelin pathway regulates cortical development, synapse formation (Tissir and Goffinet, 2003), and glutamatergic transmission (Herz and Chen, 2006), all of which are dysregulated in several psychiatric disorders. In a previous study, we showed that Reelin overexpression prevents the development of psychiatric disease-related phenotypes (Teixeira *et al*, 2011). Here we demonstrate for the first time, to our knowledge, that Dab1 downregulation during perinatal stages is sufficient to trigger structural and behavioral deficits associated with psychiatric disorders. On the basis of our findings, we propose that Dab1 developmental dysregulation is an important risk factor for the pathogenesis of such disorders. In addition, we show that D-cycloserine rescues the cognitive deficits related to Dab1 dysregulation. This finding supports the potential of this drug as a therapeutic adjunct for the treatment of cognitive deficits in psychiatric disorders for which proper treatment is still unavailable.

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The authors declare no conflict of interests.

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