Synaptojanin 1-linked phosphoinositide dyshomeostasis and cognitive deficits in mouse models of Down's syndrome

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Phosphatidylinositol-4,5-bisphosphate [PtdIns(4,5)P_2] is a signaling phospholipid implicated in a wide variety of cellular functions. At synapses, where normal PtdIns(4,5)P_2 balance is required for proper neurotransmission, the phosphoinositide phosphatase synaptojanin 1 is a key regulator of its metabolism. The underlying gene, *SYNJ1*, maps to human chromosome 21 and is thus a candidate for involvement in Down's syndrome (DS), a complex disorder resulting from the overexpression of trisomic genes. Here, we show that PtdIns(4,5)P_2 metabolism is altered in the brain of Ts65Dn mice, the most commonly used model of DS. This defect is rescued by restoring *Synj1* to disomy in Ts65Dn mice and is recapitulated in transgenic mice overexpressing *Synj1* from BAC constructs. These transgenic mice also exhibit deficits in performance of the Morris water maze task, suggesting that PtdIns(4,5)P_2 dyshomeostasis caused by gene dosage imbalance for *Synj1* may contribute to brain dysfunction and cognitive disabilities in DS.

Alzheimer's disease | inositol 5-phosphatase | phosphatidylinositol-4,5-bisphosphate | phosphatidylinositol phosphate kinase | synapse

Phosphatidylinositol-4,5-bisphosphate [PtdIns(4,5)P_2] is a key signaling phospholipid that is concentrated at the plasma membrane and controls a variety of cellular functions, including signal transduction, cell permeability, cytoskeletal dynamics, and membrane trafficking (1, 2). At neuronal synapses, where normal PtdIns(4,5)P_2 balance is required for proper neurotransmission, two brain-enriched enzymes, phosphatidylinositol phosphate kinase type 1γ (PIPK1γ) and synaptojanin 1, are key factors controlling the levels of this phospholipid (1, 3, 4). Whereas PIPK1γ synthesizes PtdIns(4,5)P_2, synaptojanin 1 dephosphorylates this lipid at sites of endocytosis and may also help to balance the action of PIPK1γ at the plasma membrane (1, 3, 4). Genetic ablation of these enzymes in the mouse leads to early postnatal lethality likely caused by defects in neurotransmission (3, 4). The critical importance of synaptojanin 1 at synapses is corroborated by genetic studies in worms, flies, and zebrafish (5–7).

A fundamental question is whether a subtle dysregulation of PtdIns(4,5)P_2 metabolism may occur in some human conditions, resulting in neurophysiological alterations and behavioral deficits. Studies have implicated the gene encoding synaptojanin 1 (*SYNJ1*) in bipolar disorder and have shown that the "mood-normalizer" lithium affects PtdIns(4,5)P_2 levels, suggesting a link between the metabolism of this lipid and some brain diseases (8). Additionally, the presence of *SYNJ1* on human chromosome 21 (HSA21) raises the possibility that it may play a role in Down's syndrome (DS). This condition, also known as trisomy 21, is the most common genetic cause of mental retardation and stems from the overexpression of some unknown number of genes present on this chromosome (9–11). Along with early development of the pathology of Alzheimer's disease and muscle hypotonia, mental retardation occurs in all DS-affected individuals, whereas other phenotypes (e.g., congenital heart defects) occur in a fraction of patients (9, 12). Although mental retardation has been linked to small subregions of HSA21, other studies have shown that the partial trisomy of nonoverlapping regions of HSA21 can also result in this phenotype (9). Thus, although evidence suggests that there is no single "mental retardation gene," a limited number of genes may contribute to the severity of this rather nonspecific phenotype. Of all 21q genes (i.e., ≈250 genes encoding ORFs >50 aa), those implicated in brain development and synaptic function are the most likely contributors to mental retardation (see http://chr21db.cudenver.edu and ref. 11 for lists).

In this study, we have investigated the potential contribution of *SYNJ1* in DS-related brain dysfunction with the anticipation that overexpression of this gene may perturb PtdIns(4,5)P_2 homeostasis at the synapse and, as a result, interfere with cognitive functions. Our data demonstrate a correlation between PtdIns(4,5)P_2 dyshomeostasis in the brain and behavioral deficits, supporting a role for *SYNJ1* in neurological manifestations in DS.

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Results

Synaptojanin 1 Overexpression in the Brain of DS Mouse Models. The expression of *Synj1* was first examined in adult Ts65Dn mice, which are segmentally trisomic for the distal portion of mouse chromosome 16 (MM16) and exhibit many features that are reminiscent of DS (13, 14). This commonly used genetic model is trisomic for a segment that is largely conserved with the long arm of HSA21, which has been previously linked to many DS anomalies; the MM16 segment contains ≈150 genes, including *Synj1* [see supporting information (SI) Fig. S1] (13, 15, 16). Western blot analysis using whole brain extracts showed a 40%

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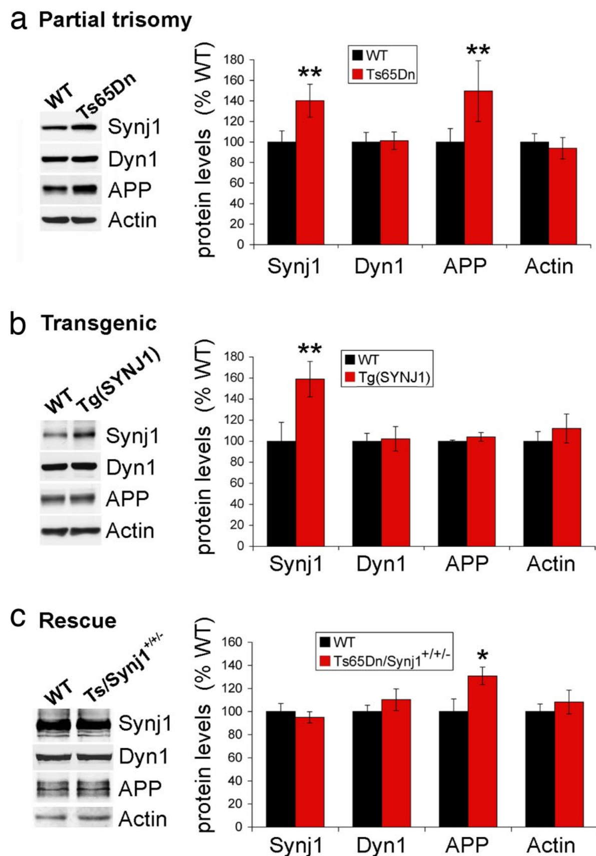

Fig. 1. Gene dosage imbalance for *Synj1* in DS mouse models. Quantitative Western blot analysis of brain extracts from Ts65Dn (*a*), transgenic mice overexpressing human *SYNJ1* (*b*), and ''rescued'' animals expressing Ts65Dn/ $Synj1^{+/+/-}$ (*c*), along with their respective controls (actin, dynamin 1). Bars denote means ± SD. Animals were 3–6 months of age. $n = 4$ for *a* and 3 for *b* and *c*. *, $P < 0.05$; **, $P < 0.01$.

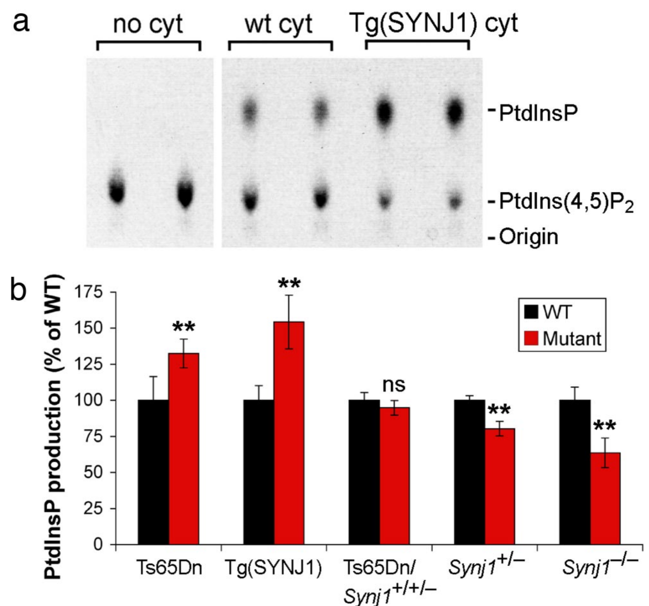

Fig. 2. *Synj1* overexpression enhances the $PtdIns(4,5)P_2$ phosphatase activity in the brain of DS mouse models. Brain cytosol extracts were used in the NBD-$PtdIns(4,5)P_2$ dephosphorylation assay. This fluorescent lipid is used as a substrate by cytosolic lipid phosphatases and converted into PtdInsP [mostly PtdIns (4)P under our assay conditions], as monitored by the use of purified standards. (*a*) Representative example with control and Tg(SYNJ1) cytosols. (*b*) Quantification of PtdInsP signals (% of total fluorescence). For Ts65Dn and the KO line ($Synj1^{+/+}$ and $Synj1^{+/-}$), $n = 4$; for Tg(SYNJ1), $n = 3$. Bars denote means ± SD. All animals were 3–5 months of age, except for $Synj1^{-/-}$ mice and their corresponding WT controls, which were newborn mice. ns, not significant. **, $P < 0.01$.

increase in the levels of synaptojanin 1 in Ts65Dn mice relative to controls ($n = 4$, $P < 0.01$) (Fig. 1*a*), thus in good agreement with predictions from gene dosage effects and previous work on human DS brain (17). We also confirmed the previously reported increase in the levels of amyloid precursor protein (APP) (13), the gene of which lies on HSA21/MM16 (Fig. 1*a* and 1*c*). No differences were found in the levels of control proteins, dynamin 1 and actin, whose genes map to different chromosomes. To understand the contribution of synaptojanin 1 in DS-linked brain dysfunction, we generated transgenic mice using BACs spanning regions that contain either human *SYNJ1* (Tg line 1) or mouse *Synj1* (Tg line 2), in combination with two unrelated and poorly characterized genes (C21orf59 and TCP10L for line 1; C21orf59 and C21orf66 mouse homologs for line 2; see Fig. S1 and Fig. S2). Because Tg line 2 contains a mouse BAC, comparison of the mRNA levels between control and Tg(Synj1) was possible. Quantitative RT-PCR analysis of whole brain mRNA extract showed that the transcript levels of *Synj1* are increased by a factor of 2.5 in Tg(Synj1), compared with controls (Fig. S2). Similar results were obtained for the neighboring gene, C21Orf59 (Fig. S1 and Fig. S2). Importantly, Western blot analyses showed a 59% and a 38% increase in the levels of synaptojanin 1 in transgenic lines 1 (Fig. 1*b*; $n = 3$, $P < 0.01$) and 2 (Fig. S2; $n = 3$, $P < 0.05$), respectively, relative to controls. Synj1 expression was returned to normal levels by genetically restoring a normal *Synj1* copy number in Ts65Dn mice (Ts65Dn/$Synj1^{+/+/-}$), which was achieved by breeding Ts65Dn mice with *Synj1* heterozygotes ($P = 0.31$; Fig. 1*c*). In contrast, the levels of APP in the brain of Ts65Dn/$Synj1^{+/+/-}$ mice remained elevated, similar to those found in Ts65Dn mice (Fig. 1 *a* and *c*). The human transgene of Tg(SYNJ1) line 1 is functional in the mouse background [Tg(SYNJ1)/$Synj1^{-/-}$] because it fully rescued the postnatal lethality phenotype of $Synj1^{-/-}$ mice (3) (Fig. S3).

PtdIns(4,5)P2 Dyshomeostasis in the Brain of DS Mouse Models. The human genome contains at least nine genes encoding inositol 5-phosphatases that can dephosphorylate $PtdIns(4,5)P_2$. Although most, if not all, of these enzymes are expressed in the brain, synaptojanin 1 represents a major contributor of inositol 5-phosphatase activity in this tissue, because its ablation greatly reduces the ability of brain extracts to hydrolyze $PtdIns(4,5)P_2$ *in vitro* (3, 4) (see also Fig. 2). To test the effect of *Synj1* overexpression on the overall $PtdIns(4,5)P_2$ phosphatase activity, brain cytosol from mutant animals and their respective controls was incubated for 15 min at 37°C with a fluorescently labeled water-soluble substrate, NBD-$PtdIns(4,5)P_2$ and conversion into NBD-PtdInsP was monitored by TLC (18) (Fig. 2). We found increases of 33% ($n = 4$, $P < 0.01$) and 54% ($n = 3$, $P < 0.01$) in the production of PtdInsP with Ts65Dn and Tg(SYNJ1) line 1 cytosol, respectively, relative to controls (Fig. 2). Comparable results were obtained with the second transgenic line (Fig. S2). Conversely, the removal of one (+/−) or two (−/−) functional *Synj1* copies led to reductions of 20% ($n = 4$, $P < 0.01$) and 36% ($n = 3$, $P < 0.01$) in the production of PtdIns4P, respectively (Fig. 2*b*). More importantly, the phosphatase activity of Ts65Dn/ $Synj1^{+/+/-}$ brains was comparable with that of WT brains ($n = 3$, $P = 0.26$; Fig. 2*b*), confirming that the increase in $PtdIns(4,5)P_2$ phosphatase activity observed in Ts65Dn brains is linked to the overexpression of a single gene, *Synj1*.

We next tested whether the *Synj1* trisomy-linked increase in

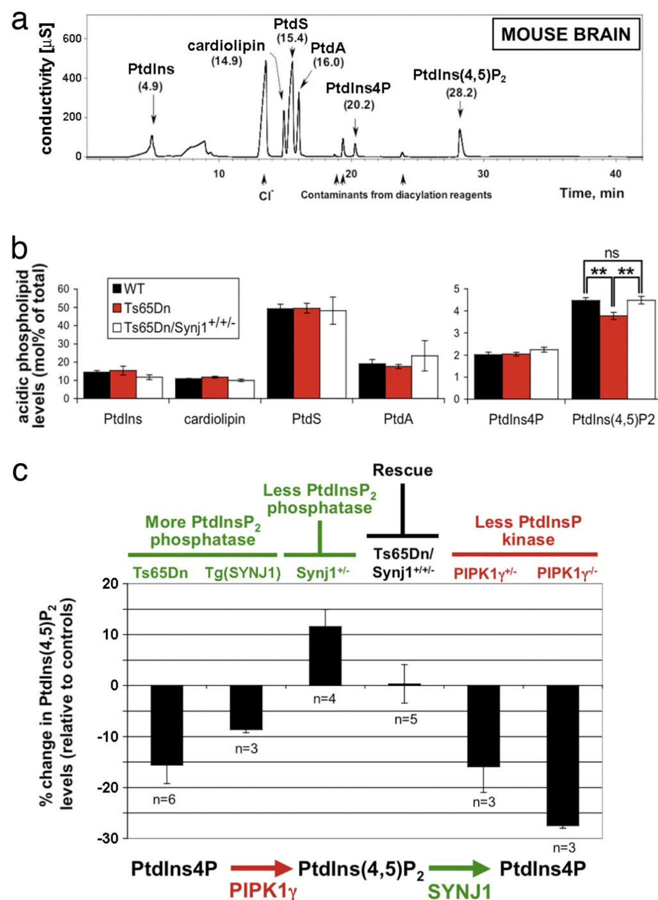

Fig. 3. Decreased PtdIns(4,5)P_2 mass in the brain of DS mouse models. HPLC combined with suppressed conductivity detection was used to measure the mass of anionic phospholipids in brain tissue. (*a*) Typical chromatogram showing the lipids detected in adult mouse brain (lipids were deacylated before analysis). Numbers in parentheses indicate retention times. (*b*) Phospholipids in normal adult mouse brain, expressed as mol % of total anionic (acidic) phospholipids. ns, not significant. **, $P < 0.01$. (*c*) Quantification of PtdIns(4,5)P_2 in the brain of mouse mutants. Bars denote means ± SEM. Animals were 3–5 months of age for Ts65Dn, Tg (SYNJ1) line 1, *Synj1*$^{+/-}$, and Ts65Dn/*Synj1*$^{+/+/-}$ (rescued). For PIPK1γ mutants, neonatal brains were used, as knockout mice die immediately after birth. SEM values for the respective control mice were: 3.6% (Ts65Dn), 1.9% [Tg(SYNJ1)], 3.4% (*Synj1*$^{+/-}$), 0.5% (Ts65Dn/*Synj1*$^{+/+/-}$), and 12.1% (*PIPK1*$\gamma^{+/-}$ and *PIPK1*$\gamma^{-/-}$).

PtdIns(4,5)P_2 phosphatase activity affects the actual mass of this lipid. Levels of phosphoinositides and other anionic phospholipids were measured and quantified in brain extracts by using HPLC with suppressed conductivity detection (19–21). A representative chromatogram of a deacylated lipid extract from mouse brain is shown in Fig. 3*a*. There was a 16% ± 4% decrease in the mass of PtdIns(4,5)P_2 in Ts65Dn brains relative to controls ($P < 0.01$, $n = 6$), with no changes in any of other anionic phospholipids measured (Fig. 3*b*). All Ts65Dn animals and their control littermates were killed between 3 and 5 months of age, thereby ruling out any contribution to this phenomenon of neurodegeneration, which typically begins after 6 months of age (22–24). Lower levels of PtdIns(4,5)P_2 were also observed in the brain of transgenic mice overexpressing *Synj1* ($9 \pm 1\%, P < 0.01$, $n = 3$) (Fig. 3*c*). Conversely, there was a 12% ± 3% increase in the levels of PtdIns(4,5)P_2 in the brain of *Synj1*$^{+/-}$ mice ($n = 4$, $P < 0.05$) (Fig. 3*c*). Because there was a trend for a more severe PtdIns(4,5)P_2 deficiency in Ts65Dn relative to transgenic brains, other factors may contribute to this phenomenon in trisomic brains (see *Discussion*). However, the removal of one functional *Synj1* copy from Ts65Dn mice fully corrected the PtdIns(4,5)P_2 defect (WT vs. rescued, not significant, $P = 0.48$) (Fig. 3*c*), indicating that the overexpression of synaptojanin 1 mediates this defect in the brain of trisomic mice. For comparison, levels of PtdIns(4,5)P_2 were determined in neonatal brain from *PIPKI*$\gamma^{+/-}$ and *PIPKI*$\gamma^{-/-}$ mice in which lower synthesis, rather than increased dephosphorylation, of this lipid occurs. There were decreases of 16% ± 5% and a 28% ± 1% in the levels of PtdIns(4,5)P_2 in *PIPKI*$\gamma^{+/-}$ and *PIPKI*$\gamma^{-/-}$ brains, respectively ($n = 3$, $P < 0.05$ for −/−) (see also ref. 4). Thus, the PtdIns(4,5)P_2 deficiency in trisomic mice is comparable with that found in *PIPKI*$\gamma^{+/-}$ mice (Fig. 3*c*).

Next, based on the reported synaptic localization of synaptojanin 1 (25), we investigated whether PtdIns(4,5)P_2 metabolism is specifically altered in nerve terminals. Cortical synaptosomes from adult control and Ts65Dn mice were incubated for 30 min with [^{32}P] inorganic phosphate to achieve metabolic labeling of phospholipids, such as PtdA, PtdInsP, and PtdInsP_2. In resting Ts65Dn synaptosomes, the PtdInsP_2/PtdA ratio was decreased by ≈30% relative to controls, consistent with increased PtdIns(4,5)P_2 hydrolysis (Fig. S4). However, high K^+-induced depolarization slightly attenuated the difference in PtdInsP_2 labeling (see Fig. S4). Altogether, our biochemical results strongly suggest that PtdIns(4,5)P_2 metabolism is altered in the brain of Ts65Dn mice and that a major underlying cause of this phenomenon is *Synj1* trisomy.

Learning Deficits in Transgenic Mice Overexpressing Synaptojanin 1. Mental retardation is a consistent primary phenotypic manifestation of DS (12) and mouse models for this disorder, such as Ts65Dn animals, exhibit learning deficits in a variety of behavioral tasks (13, 14). Although these deficits have a multigenic origin, a major challenge is to understand the relative contribution of individual genes to this phenomenon. To this aim, we conducted behavioral analyses on the transgenic mouse model Tg(SYNJ1) line 1 by using the Morris water maze paradigm, based on previous reports showing that Ts65Dn animals exhibit spatial learning deficits in this task (13, 26). In this paradigm, animals are first tested in the visible platform task. Individual mice are placed in a swimming pool and are required to locate a platform that is marked by a flag. This test can unmask defects in swimming ability, motivation, vision, and cognitive deficits that are not restricted to spatial learning. Next, animals are evaluated in the hidden platform test in which they are trained to locate an invisible platform in the pool by using spatial cues that are present in the environment. After several days of training, the platform is removed and animals are allowed to swim freely for 60 s (probe test). The time spent in each of the four quadrants is recorded and, typically, animals that have learned spend more time in the quadrant that originally contained the platform (TQ).

Results from the visible platform test showed no obvious differences in the escape latency (i.e., the time required to find the platform) between the two genotypes (Fig. S5). Animals were then trained to perform the hidden platform task in a series of experiments that also allowed us to assess the impact of the training strength on learning. First, animals were subjected to a limited training phase with only one session per day in a environment containing a low number of spatial cues. The escape latency did not significantly decrease [$F(1,27) = 0.28, P = 0.94$] over the course of seven sessions for both genotypes (Fig. S5). However, control animals had clearly learned the task, because they spent more time in the TQ compared with the other three quadrants in the probe test ($P < 0.001$ for opposite quadrant and $P < 0.05$ for the adjacent quadrants) (Fig. 4*a*). On the contrary, Tg(SYNJ1) animals did not learn the task, as they spent a comparable fraction of their swimming time in all four quadrants ($P > 0.2$) (Fig. 4*a*). Given the limited training, the

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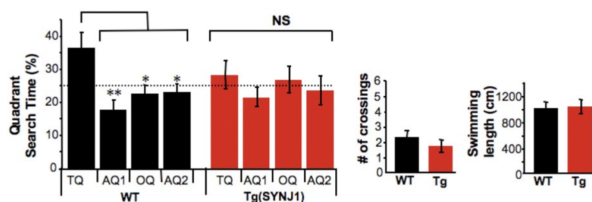

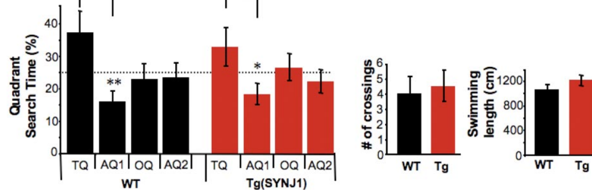

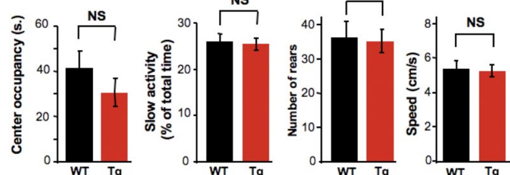

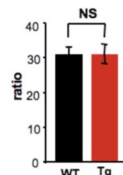

Fig. 4. Transgenic mice overexpressing synaptojanin 1 (line 1) exhibit learning deficits in the Morris water maze task. (*a*) Results of the probe trial after a limited training hidden platform protocol with 16 WT (5 males and 11 females) and 13 Tg(SYNJ1) (4 males and 9 females) mice. (*Left*) Control mice spent more time in the TQ compared with the adjacent quadrants ($P < 0.01$ and $P < 0.05$ for AQ1 and AQ2, respectively) and the opposite quadrant (OQ; $P < 0.05$), whereas Tg(SYNJ1) mice spent equal time in all four quadrants, thereby showing no learning ($P > 0.05$). (*Center*) Number of crossings over the area that originally contained the platform during the probe trial. (*Right*) Swimming path length (cm). (*b*) Results of the probe trial after an extensive hidden platform training protocol using 9 WT (2 males and 7 females) and 12 Tg(SYNJ1) (4 males and 8 females) mice, showing no differences in quadrant occupancy between the genotypes. Other parts of the test are shown in Fig. S5. (*c*) Open-field test showing no differences between control ($n = 8$) and transgenic mice ($n = 7$) in the four indicated parameters. *P* values were 0.28 (center occupancy), 0.81 (slow activity), 0.87 (rears), and 0.8 (speed), respectively. (*d*) Elevated plus maze test showing no difference ($P = 0.98$) between control (31.0 ± 2.0%, $n = 12$) and transgenic mice (31.1 ± 2.8%, $n = 10$). The ratio between the number of entries in the open arms over that in the closed arms was calculated. Results from *c* and *d* suggest that anxiety-related behavior is normal in the transgenic mice from line 1. Values denote the means ± SEM. **, $P < 0.01$; *, $P < 0.05$; NS, not significant.

number of platform crossings was low for both genotypes, although there was a trend toward a higher number of platform crossings for controls relative to transgenic mice (Fig. 4*a*). There was no genotype-specific difference in the swimming path length during the probe test (Fig. 4*a*).

In a second set of experiments, we subjected the animals to an extensive training regimen consisting of two sessions a day (instead of one) and with a pool environment enriched with visual cues. Both control and transgenic mice improved their performance over the course of days in the hidden platform task [$F(1,19) = 7.1$, $P < 0.0001$] with no notable difference between genotypes [$F(1,19) = 0.02$, $P = 0.90$; Fig. 4*b*]. Similarly, the probe test revealed that both genotypes had learned the task comparably, because they showed a tendency to prefer TQ over the other three quadrants ($P < 0.05$ for AQ1). The number of platform crosses and the swimming path length were comparable for both genotypes ($P = 0.64$ and 0.24, respectively; Fig. 4*b*). Thus, results indicate that the transgenic mice have learning deficits in the Morris water maze, requiring a more thorough training protocol to achieve the same level of performance as controls.

We next examined an independent line of transgenic mice, the Tg(Synj1) line 2, whose genetic background differs from that Tg(SYNJ1) line 1 (see *Materials and Methods*). In contrast to Tg(SYNJ1) line 1 (Fig. 4), these mice showed no deficit compared with controls in the limited training protocol. Because of this lack of deficit, we did not test them further with the enhanced protocol. Instead, we examined their performance in the reverse platform test, which is a variation of the Morris water maze paradigm where animals are required to learn a new platform location after the probe trial and are thus tested for their ability to renew their spatial memory. A failure to adapt to new tasks is a feature reminiscent of Ts65Dn animals (27). In this task, Tg(Synj1) line 2 did not perform as well as control animals, as shown by their longer escape latencies in the hidden platform task relative to controls [$F(1,17) = 5.75$, $P = 0.019$] (Fig. S6).

Anxiety-related behavior is known to affect the learning performance in rodents. To rule out this parameter as a potential confounding factor for the interpretation of the Morris water maze experiments, animals were first subjected to the open-field test (28). In this paradigm, mice are placed in a novel and bright arena. As the animals naturally tend to avoid brightly illuminated areas, locomotor activity and exploratory behaviors are believed to reflect the level of anxiety undergone by the animals. Results from Fig. 4*c* and Fig. S6 show that both lines of transgenic mice behave similarly to their respective control group based on various parameters tested. Next, animals were subjected to the elevated plus maze test, which relies on rodents' tendency toward dark, enclosed spaces and an unconditioned fear of heights/open spaces Because the elevated open alleys produce a strong approach–avoidance conflict, animals will tend to prefer the enclosed alleys (29). Mice are placed at the center of a plus-shaped apparatus that includes two open and two enclosed elevated alleys and are allowed to walk freely in the maze for 10 min. Anxiety-related behavior is assessed by calculating the ratio between the number of entries into the open arms and that into the closed arms. Fig. 4*d* and Fig. S6 show that this ratio was comparable between control and transgenic mice from both lines. In conclusion, transgenic animals do not appear to exhibit higher anxiety-related behavior relative to controls, suggesting that differences in this type of behavior are unlikely to account for the observed differences in learning between genotypes.

Discussion

Based on a growing number of studies pointing to a critical role of synaptojanin 1 and, more generally, PtdIns(4,5)P_2 metabolism, in synaptic function and on the presence of *SYNJ1* on HSA21 (3–7, 30, 31), we have investigated the contribution of this gene to DS-linked brain dysfunction. Although HSA21 contains a fairly large number of genes that could contribute to neurological phenotypes in DS, a specific effect of individual genes is plausible, as suggested for instance by the implication of *App* trisomy in cholinergic neurodegeneration and endosomal abnormalities in Ts65Dn mice (22–24). Here, we have shown in three different trisomy models that the overexpression of synaptojanin 1 causes a defect in the metabolism of PtdIns(4,5)P_2 in the brain. Importantly, restoring selectively the disomy of *Synj1* in the Ts65Dn mouse also re-establishes normal PtdIns(4,5)P_2 levels. Consistent with a gene dosage effect, the biochemical defect caused by *Synj1* overexpression is subtle and does not appear to impact viability, in contrast to the more severe alterations of PtdIns(4,5)P_2 metabolism seen in *PIPK1γ* and *Synj1* knockout mice (3, 4). The fact that Synj1 overexpression produces a decrease in the mass of PtdIns(4,5)P_2 in trisomic brains indicates that homeostatic mechanisms fail to correct this

metabolic imbalance. A possible explanation is that the pool of PtdIns(4,5)P_2 hydrolyzed by synaptojanin 1 may be in part spatially segregated from that controlled by PtdIns(4,5)P_2-synthesizing enzymes. Although PtdIns(4,5)P_2 is the only anionic phospholipid shown to be altered in the DS models, local changes in PtdIns(3,4,5)P_3 (i.e., another substrate of the inositol 5-phosphatase domain) or in the putative substrates of synaptojanin 1's Sac1 domain, such as PtdIns3P and PtdIns4P, cannot be ruled out (32). Additionally, dominant negative effects resulting from the overexexpression of *Synj1* and a perturbation of its Src homology 3 domain-containing interactors cannot be excluded, although the subtle gene dosage imbalance caused by trisomy makes this possibility unlikely.

Because PtdIns(4,5)P_2 plays pleiotropic roles in cells (1, 2), anomalies in its metabolism may interfere with a large number of cellular functions. However, the predominant localization of synaptojanin 1 at synapses (25) suggests that the imbalance of PtdIns(4,5)P_2 may have an impact at these sites. Studies carried out in various organisms have provided robust evidence for a role of synaptojanin in synaptic vesicle recycling and neurotransmitter receptor (e.g., AMPA receptor) internalization, consistent with the importance of PtdIns(4,5)P_2 in the assembly of endocytic complexes at the plasma membrane (3, 5–7, 30, 31, 33). The function of ion channels, transporters, and a variety of other key components of synaptic membranes may be affected by abnormal PtdIns(4,5)P_2 levels (2, 34). Additionally, perturbation of synaptojanin function affects the dynamics of actin (35), which is critical for normal basal neurotransmission and synaptic plasticity in many instances. Thus, the overexpression of synaptojanin 1 may affect a variety of synaptic processes, particularly in brain regions normally exhibiting the highest levels of expression, such as the hippocampus (25). However, electrophysiological analysis of hippocampal slices from control and transgenic mice did not reveal significant changes in basal neurotransmission and synaptic plasticity (e.g., long-term potentiation and paired-pulse facilitation) in the two transgenic lines, suggesting that neurotransmission defects may be subtle at best (Fig. S7). Synaptojanin 1 overexpression may also cause developmental defects, as this gene is expressed in the brain starting from midembryonic life.

Based on published studies of the Ts65Dn mice and our work on transgenic animals, we can compare performance in the Morris water maze in three different models expressing comparable levels of Synj1, but carrying different sets of trisomic genes. Transgenic mice carrying the human *SYNJ1* BAC (line 1) learn poorly in the Morris maze when subject to a limited training protocol, but learn normally when a more extensive training protocol is used. This finding suggests that (acute) environmental enrichment and increasing the frequency of training sessions confer neurobehavioral benefit to these mutant animals and allow them to overcome transgenic effects. Abnormal anxiety-related behavior is unlikely to mediate the deficits in performance of transgenic mice in the Morris maze task, because the latter performed similar to controls in the open field and elevated plus maze tests. Transgenic mice carrying the mouse *Synj1* BAC (line 2) and one additional gene shared in common with the BAC from line 1 learn normally even in the limited training protocol, but learn poorly in the reverse location protocol. Differences in learning behavior between the two transgenic lines may stem from variations of the genetic background and gene content (see Fig. S1). Strikingly, compared with both transgenic lines, the learning deficits of Ts65Dn mice in the Morris maze are more severe (26), consistent with the general concept that neurological defects in DS and genetic models thereof result from contributions of multiple genes. Some of these may act in concert with *Synj1* to perturb synaptic function by affecting overlapping biochemical pathways. Such genes include *ITSN1*, *DYRK1a*, and *DSCR1*, which encode, respectively: (*i*) intersectin 1, a direct interactor of synaptojanin 1 (36); (*ii*) Dyrk1A/minibrain kinase, a protein kinase that phosphorylates synaptojanin 1 (37); and (*iii*) Dscr1, a regulator of the protein phosphatase calcineurin, which mediates the dephosphorylation of synaptojanin 1 during nerve terminal depolarization (18, 38) (see also ref. 39. for the evidence of genetic interaction between *DYRK1a* and *DSCR1* in DS models). To unambiguously assess the contribution of *Synj1* overexpression to cognitive deficits characteristic of Ts65Dn mice, it will be essential to analyze the learning performance of Ts65Dn mice containing a normal copy number of *Synj1*.

Altogether, our study on genetic models of DS strongly suggests that anomalies in PtdIns(4,5)P_2 metabolism may contribute to neuronal dysfunction and cognitive deficits in individuals with this disorder. Future work should address whether a perturbation of this lipid is also involved in cognitive decline, paralleling the development of Alzheimer's disease in individuals with DS (23, 24). Indeed, a recent study has suggested a link between PtdIns(4,5)P_2 dyshomeostasis and Alzheimer's disease-associated mutations of presenilins, i.e., the components of the γ-secretase complex responsible for the generation of amyloid-β (Aβ) peptide (40). Furthermore, acute and chronic treatments of primary cortical neurons with soluble oligomers of Aβ 1-42 (i.e., the most potent synapse-impairing assembly of the peptide) have been shown to down-regulate PtdIns(4,5)P_2 levels (21). Because *Synj1* haploinsufficiency suppresses the deleterious effects of Aβ 1-42 on hippocampal LTP, Aβ-induced synaptic impairment may occur, at least in part, through a perturbation of PtdIns(4,5)P_2 metabolism (21). Thus, the brain of individuals with DS may undergo a dual "hit" on PtdIns(4,5)P_2, accounted for by *Synj1* trisomy, as shown in this study, and by Aβ elevation, which could accelerate Alzheimer's disease-linked cognitive decline in middle-aged adults with DS.

Materials and Methods

Animal Models. Ts65Dn animals were obtained from The Jackson Laboratory (13). These animals are maintained on a segregating background by backcrossing Ts65Dn females to C57BL/6JEi $\times$ C3H/HeSnJ (B6EiC3Sn) F_1 males and are genotyped as described (41). Tg(SYNJ1) line 1 was generated on the C57BL/6J background at The Jackson Laboratory by using human BAC RPCI13–412C15, which was identified by PCR screening of the RPCI13 library. Tg(Synj1) line 2 was generated on the FVB background by using mouse BAC RPCI-23 402J16. Because of early-onset retinal degeneration in this strain, FVB mice were bred with C57BL/6 mice, and F_1 hybrids were used for behavioral analyses. For the genotyping of the two transgenic lines, PCR amplifications of the 5′ and 3′ ends of the BAC were performed. *Synj1*$^{+/-}$ mice have been described (3), although for the current study, animals in their original background (mixed 50:50 129Sv/C57BL/6J) were first backcrossed into the C57BL/6 background for six generations and subsequently bred with C3H/HeSnJ mice to obtain B6C3Sn F_1 hybrids and match the background of Ts65Dn mice.

Biochemistry. Western blot analyses of brain tissue from 3- to 5-month-old mice were performed as described (3) by using rabbit polyclonal antibodies to the COOH termini of synaptojanin 1, dynamin 1, and APP or mouse monoclonal antibody AC-15 to beta-actin (Sigma–Aldrich) (40). Secondary antibodies were either coupled with HRP for ECL detection (GE Healthcare) or IR dyes for IR signal detection (Rockland). For the quantification of ECL protein signals, films were scanned and bands were analyzed by using optical densitometry and Image J software. For IR immunoblots, membranes were exposed on an Odyssey IR scanner, and band intensities were quantified by using the software from the Odyssey imaging system (Li-Cor Biosciences). Comparable results were obtained with both approaches. Quantification of transcript levels in the brain of transgenic mice was performed as described (16). The PtdIns(4,5)P2 phosphatase assay was performed as in ref. 18 with brain cytosol (1 μg) instead of purified enzymes. The quantification of anionic phospholipids from brain tissue was performed by anion-exchange HPLC as described (19–21, 40).

Behavioral Analysis. Three- to four-month-old mice were used for all of the experiments. The Morris water maze was performed as described (42) with some modifications in the hidden platform test. Two protocols were used: (*i*)

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a limited training protocol, which consisted of only one session per day with few visual cues, and (*ii*) an extensive training protocol, which consisted of two sessions per day (4-h interval between the sessions) with a higher number of highly distinguishable cues located on the wall of the pool. All experiments were performed blind with respect to the genotypes. Separate tests were also performed for males and females. Because no sex-specific differences were found, results from both genders were pooled in all our experiments. For the open-field test (28), mice were placed in the same corner of the box (a brightly illuminated 42 $\times$ 34-cm rectangle arena) and allowed to freely explore for 10 min. The ''center'' field was defined as the central 20 $\times$ 16-cm area of the open field, $\approx$22% of the total area. The total time spent in the center, the number of rears, the speed, and the proportion of slow activity (defined as immobility and walking inferior to 1 cm/s) were measured. For the elevated plus maze, the plus maze was made of four dark gray Plexiglas arms: two open arms (67 $\times$ 7 cm), and two enclosed arms (67 $\times$ 7 $\times$ 17 cm) that formed a cross shape with the two open arms opposite to each other. The maze was 55 cm above the floor and dimly illuminated. Mice were placed individually on the central platform, facing an open arm, and allowed to explore the apparatus for 10 min. Anxiety was assessed by the ratio between the number of entries into the two open arms over the two closed arms (29).

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