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Neurodevelopmental delay, motor abnormalities and cognitive deficits in transgenic mice overexpressing *Dyrk1A* (*minibrain*), a murine model of Down's syndrome

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Down's syndrome (DS) is a major cause of mental retardation, hypotonia and delayed development. Murine models of DS carrying large murine or human genomic fragments show motor alterations and memory deficits. The specific genes responsible for these phenotypic alterations have not yet been defined. *DYRK1A*, the human homolog of the *Drosophila minibrain* gene, maps to the DS critical region of human chromosome 21 and is overexpressed in DS fetal brain. *DYRK1A* encodes a serine-threonine kinase, probably involved in neuroblast proliferation. Mutant *Drosophila minibrain* flies have a reduction in both optic lobes and central brain, showing learning deficits and hypoactivity. We have generated transgenic mice (TgDyrk1A) overexpressing the full-length cDNA of *Dyrk1A*. TgDyrk1A mice exhibit delayed craniocaudal maturation with functional consequences in neuromotor development. TgDyrk1A mice also show altered motor skill acquisition and hyperactivity, which is maintained to adulthood. In the Morris water maze, TgDyrk1A mice show a significant impairment in spatial learning and cognitive flexibility, indicative of hippocampal and prefrontal cortex dysfunction. In the more complex repeated reversal learning paradigm, this defect turned out to be specifically related to reference memory, whereas working memory was almost unimpaired. These alterations are comparable with those found in the partial trisomy chromosome 16 murine models of DS and suggest a causative role of *DYRK1A* in mental retardation and in motor anomalies of DS.

INTRODUCTION

Trisomy 21 or Down's syndrome (DS) is a major cause of mental retardation (1). Hypotonia, early-onset Alzheimer's disease and behavioral alterations are also constant in DS patients. The central nervous system (CNS) of DS patients shows a large number of alterations, including a reduction of brain size, abnormal neuronal migration, differentiation and density, affecting several brain regions, and abnormal dendritic arborization, among other changes. The cellular, biochemical and molecular bases of these CNS alterations are unknown.

Which genes of the 225 genes of human chromosome 21 (HSA21) catalog (2) are responsible for the CNS alterations of DS? Exceptional cases of DS are due to partial trisomy 21 and have allowed the definition of a critical region (DSCR) that, if triplicated, is associated with numerous clinical features, including mental retardation and hypotonia (3,4). Although challenged by rare cases of trisomy affecting other parts of the chromosome (5), this critical region between markers *D21S17* and *ETS2* has attracted the interest of many investigators and focused intense effort in gene characterization (6).

One of the DSCR genes is *DYRK1A/MNBH*, which is the human homolog of the *Drosophila minibrain* gene (7). Mutant *minibrain* flies have reduced optic lobes and central brain and show learning deficits and hypoactivity (8,9). *DYRK1A* encodes a serine-threonine kinase (10), which is expressed during neuroblast proliferation in *Drosophila* (11). Expression of *DYRK1A* is detected in several regions of the CNS, from development to adulthood, especially in the cortex, hippocampus and cerebellum (7,12). Overexpression of *DYRK1A* has been found in DS fetal brain and its murine homolog *Dyrk1A* is also overexpressed in Ts65Dn mice (12), a murine model partial trisomic for mouse chromosome 16 (MMU16) (13).

Murine models of DS carrying large murine or human genomic fragments show motor alterations and memory

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

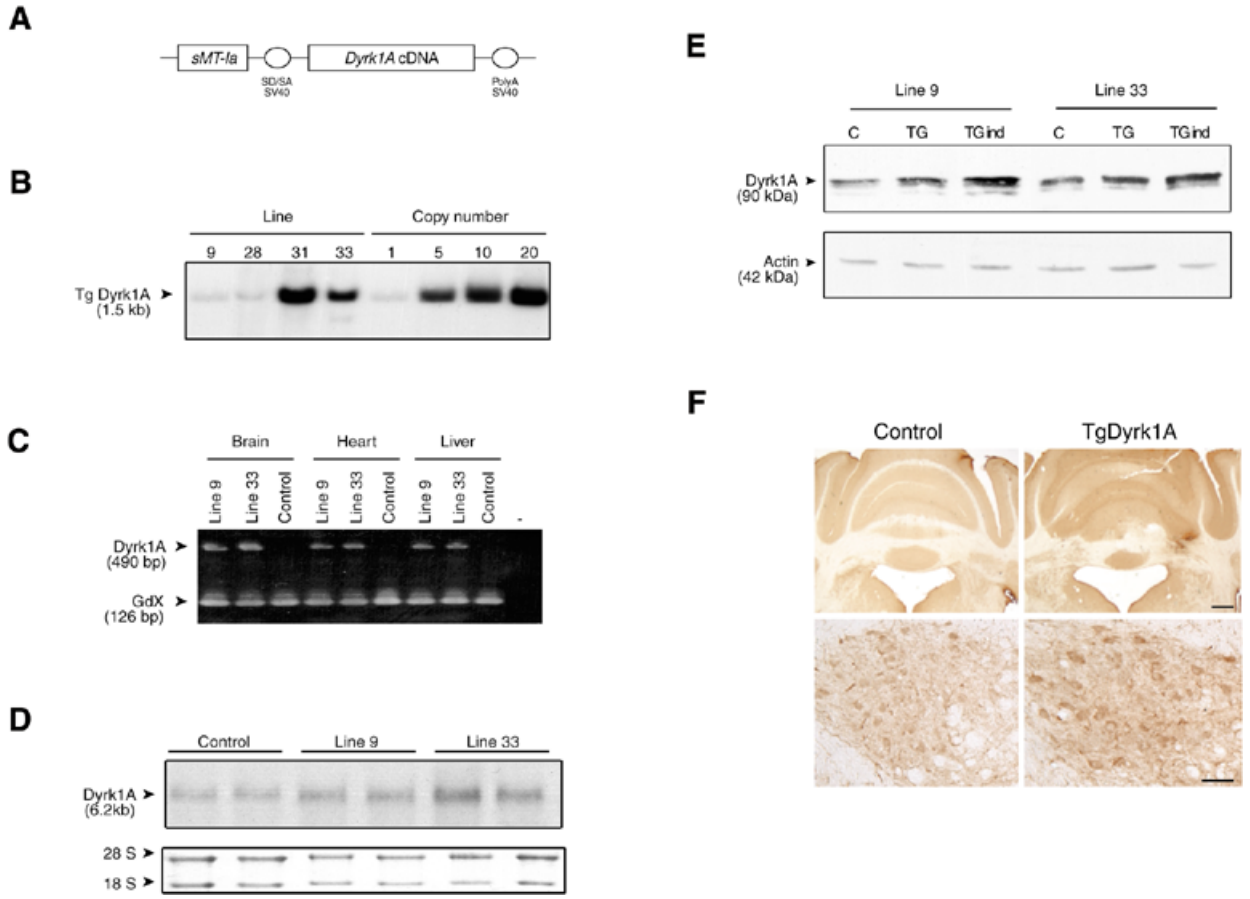

Figure 1. Genotyping and expression analysis of TgDyrk1A mice. (**A**) Schematic representation of the *sMT-Ia/Dyrk1A* chimeric gene. (**B**) Southern blot analysis of four independent transgenic lines (L9, L28, L31 and L33) carrying two, two, 20 and five copies of the transgene, respectively. (**C**) Expression of the transgene by RT–PCR analysis in brain, heart and liver of TgDyrk1A mice L9 and L33 and control mice. (**D**) Northern blot analysis from RNA isolated from adult brain in control and TgDyrk1A mice, L9 and L33. (**E**) Western blot analysis from adult brain of control, transgenic, and transgenic mice induced with $ZnSO_4$ injection (20 mg/kg, intraperitoneal) 24 h before death. (**F**) Immunohistochemistry analysis of TgDyrk1A mice (L9) 24 h after induction with $ZnSO_4$ injection, and wild-type littermate. Immunoreactivity is present in the cerebellar cortex (top bar, 500 µm) and the medial cerebellar nuclei (bottom bar, 50 µm), in control and induced TgDyrk1A (L9). Notice a generalized increase in Dyrk1A immunoreactivity in the grey matter of the cerebellar cortex (top) and the cytoplasm of selected neurons in the medial cerebellar nuclei (bottom) of TgDyrk1A (L9) after $ZnSO_4$ induction.

deficits (13–18). Transgenic mice with a yeast artificial chromosome (YAC) containing the human *DYRK1A* gene exhibited at least some alterations analogous to those found in DS pathology, such as memory deficits (13). In an effort to evaluate the contribution of *DYRK1A* overexpression to DS, we have generated transgenic mice overexpressing specifically the full-length cDNA of *Dyrk1A* under the control of the inducible sheep metallothionein-Ia (*sMT-Ia*) promoter. TgDyrk1A mice exhibit alterations in neuromotor development, hyperactivity, and significant impairment in spatial learning and memory. All these results suggest a causative role of *DYRK1A* in mental retardation and motor anomalies of DS.

RESULTS

Generation of transgenic mice overexpressing *Dyrk1A*

The *sMT-Ia/Dyrk1A* chimeric gene used to obtain transgenic mice is shown in Figure 1A. Four founder mice, carrying between two and 20 copies of the transgene, were obtained after microinjection, as confirmed by Southern blot analysis (Fig. 1B), and four transgenic lines, designated as lines 9, 28, 31 and 33, were established. Transgenic lines 9 and 33 (L9 and L33) were used for all the experiments.

To determine expression of the transgene, RT–PCR analysis of different tissues was performed showing ubiquitous expression of the transgene (Fig. 1C). Northern blot analysis from adult brain RNA demonstrated the presence of a 6.2 kb transcript corresponding to Dyrk1A. The levels of this transcript were higher in the transgenic mice, ~1.5- and 2.2-fold, in L9 and L33, respectively (Fig. 1D). Overexpression of the transgene was also detected by western blot analysis. This overexpression was increased after induction by intraperitoneal injection of 20 mg/kg of $ZnSO_4$ (Fig. 1E). TgDyrk1A and control mice showed a similar Dyrk1A immunostaining pattern in the CNS. Overexpression was mild under basal conditions and was increased after induction of the transgene expression by $ZnSO_4$ (Fig. 1F). Immunoreactivity for Dyrk1A presented an overall increase in the brain, essentially in grey matter, although immunoreactive glial cells were also detected.

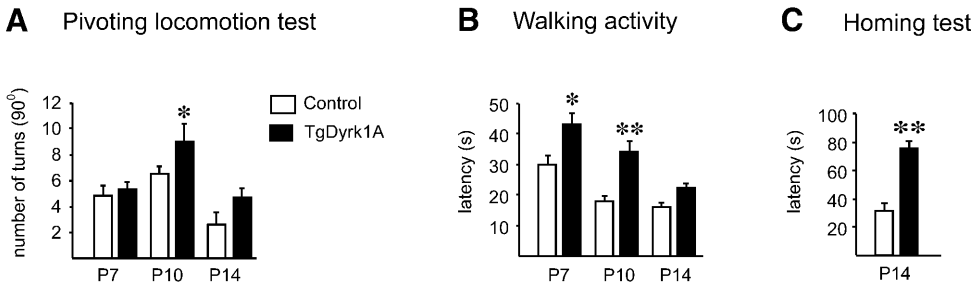

Figure 2. Neuromotor development in TgDyrk1A mice (L9 and L33, $n = 14$, filled bars) and wild-type mice ($n = 11$, open bars). (**A**) TgDyrk1A mice showed persistence of immature locomotor patterns compared with control littermates. (**B**) TgDyrk1A mice showed delayed walking activity. At P7 and P10, the latency to initiate walking was significantly increased in transgenic versus control mice. At P14 the latency was more importantly reduced in TgDyrk1A than in control mice. (**C**) Retarded general psychomotor development in TgDyrk1A mice. At P14, the latency to reach the goal arena in the homing test was higher in transgenic than in control mice. Data are expressed as mean ± SEM. $*P < 0.05$, $**P < 0.01$; Student's *t*-test.

Neuropathological analysis in TgDyrk1A

TgDyrk1A ($n = 4$) and control ($n = 4$) mice were killed for neuropathological examination. There were no differences in mean brain weight, and the histological analysis showed no gross alterations in the CNS of TgDyrk1A mice. No obvious signs of gross malformations or inflammatory, ischemic or hypoxic changes in the hematoxylin and eosin (H&E) stained sections were observed (data not shown).

Neurodevelopmental analysis of TgDyrk1A mice

Developmental studies were performed in transgenic mice from L9 and L33 ($n = 14$) and wild-type littermates ($n = 11$). Reproductive performance and litter viability were unaffected. TgDyrk1A were normal in their somatometric development and in the emergence of developmental landmarks. No alterations in the maturation of sensorial and reflexologic responses were observed. Despite this, TgDyrk1A mice showed retardation in neuromotor development, characterized by the persistence of immature locomotor patterns and a delay in maturation of gait. In the pivoting test (Fig. 2A), the number of turnings at P10 was significantly increased in TgDyrk1A mice compared with control mice ($t = 3.6$, $P < 0.05$; Student's *t*-test). The delayed acquisition of mature locomotor activity was shown by the increased latency to initiate walking activity at P7 ($t = 4.6$, $P < 0.05$; Student's *t*-test) and P10 (Fig. 2B). In normal conditions the latency to initiate walking decreases progressively as the cranio-caudal development is completed. However, the reduction in latency at P14 with respect to P10 was significantly less important in TgDyrk1A mice than in control mice ($t = 6.75$, $P < 0.018$; Student's *t*-test). Thus, our results suggest a delay in the acquisition and maturation of locomotion from cranial to caudal parts of the body, specifically related to gait. TgDyrk1A mice presented a retardation in general development in the homing test (P14), where an increased latency to reach the goal arena was observed with respect to control ($t = 7.1$, $P < 0.01$; Student's *t*-test) (Fig. 2C).

TgDyrk1A mice (L9 and L33, $n = 22$) also exhibited alterations in the wire suspension test, an index of motor coordination during neurodevelopment, as shown by the significantly lower latency to fall with respect to control littermates ($n = 13$) at P14 [$F_{(2,35)} = 4.25$, $P = 0.024$; analysis of variance (ANOVA)] (Fig. 3A).

Table 1. Alterations of motor activity and emotionality in TgDyrk1A mice

	Control	Tg Dyrk1A	
Elevated plus maze test (dark phase)			
Number of entries in the open arms	3.9 ± 0.3	5.8 ± 0.6	***
Distance crossed in the open arms (m)	2.6 ± 0.3	4.2 ± 0.5	**
Time in the open arms (%)	9.8 ± 1.1	14.1 ± 4.6	*
Speed in the open arms (cm/s)	8.3 ± 0.5	8.6 ± 0.6	n.s.
Open field activity (light phase)			
Number of crossings in the center	16.3 ± 2.4	25.1 ± 2.3	**
Number of crossings in the periphery	58.2 ± 4.1	69.6 ± 3.6	*
Time in the center (%)	6.3 ± 1.0	9.1 ± 0.7	*
Time in the periphery (%)	86.4 ± 1.5	81.7 ± 1.3	*

In the open field and plus maze tests, TgDyrk1A (L9 and L33, $n = 28$) showed mild hyperactivity and reduced emotionality with respect to control mice ($n = 23$).
Data are expressed as means ± SEM. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; n.s., not significant; Student's *t*-test.

Behavioral characterization in adult TgDyrk1A mice

The motor impairment observed during development was maintained to some extent in adulthood. Adult TgDyrk1A mice exhibited a reduction in the latency to fall in the coat hanger test ($t = 3.0$, $P < 0.01$; Student's *t*-test) (Fig. 3B). Moreover, the number of crossings along the bar was reduced in transgenic mice ($t = 2.7$, $P = 0.01$; Student's *t*-test). There was no detectable line effect along the motor characterization.

Adult TgDyrk1A showed alterations in general locomotor activity (Table 1). In the absence of differences in circadian activity as measured in the actimetry test ($t = 0.6$, $P = 0.56$, not significant), which provided an estimate of baseline activity in a familiar environment, there was a consistent finding of increased locomotion in a novel environment along different test conditions. In the open field test (light phase) TgDyrk1A mice showed mild hyperactivity as shown by the increased number of crossings in the periphery of the arena ($t = 2.1$, $P < 0.05$). This increased locomotor activity was also observed in the center of the arena ($t = 2.7$, $P = 0.01$). TgDyrk1A mice spent significantly more time in the center of the arena ($t = 2.4$, $P < 0.05$)

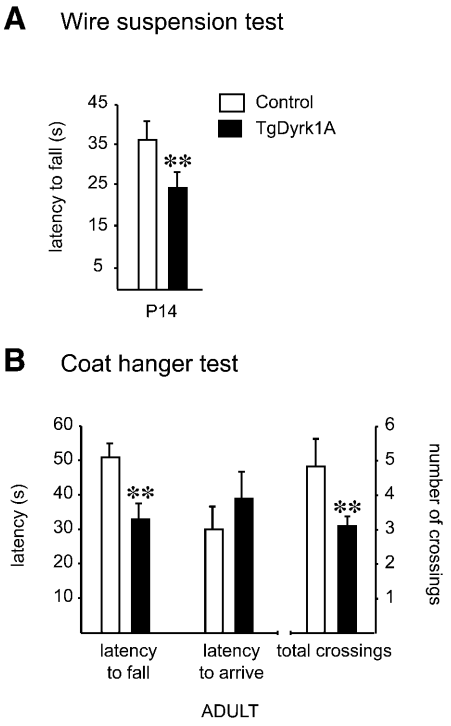

Figure 3. Motor coordination in TgDyrk1A (L9 and L33, $n = 22$, filled bars) and wild-type ($n = 13$, open bars) mice. (**A**) At P14, the latency to fall in the wire suspension test was smaller in transgenic than in control mice. (**B**) In adult life motor coordination remained altered in transgenic mice in the coat hanger test as shown by the lower latency to fall in TgDyrk1A versus control mice. Moreover, the number of crossings along the bar was reduced in transgenic mice. Data are expressed as means ± SEM. $^*P < 0.05$, $^{**}P < 0.01$.

than on the periphery ($t = 2.4$, $P < 0.05$), suggesting a reduced emotional response in transgenic mice. This result was confirmed in the elevated plus maze (dark phase) where TgDyrk1A mice also exhibited hyperactivity, as demonstrated by the increase in the total number of entries in open and enclosed arms (Table 1) and reduced emotionality. The later effect was shown by the increase in the number of entries ($t = 3.1$, $P < 0.01$) and in the distance traveled in open arms ($t = 2.8$, $P < 0.01$). The effect is accompanied by an increase in the percentage of time spent in the open arms ($t = 2.6$, $P < 0.05$). Hyperactivity is not due to an increased speed of transgenic mice since this parameter is not altered ($t = 0.3$, $P = 0.77$, not significant).

Performance of TgDyrk1A and wild-type mice in the Morris water maze

The first experiment in the Morris water maze, using a spatial reference memory procedure, showed that both control and transgenic mice (L33) learned to reach the platform during acquisition, but the learning curves were significantly different [genotype effect $F_{(1,11)} = 5.35$; $P = 0.041$]. Repeated measures ANOVA on the escape distances in the hidden platform test revealed a significant effect of session both in wild-type and TgDyrk1A mice [$F_{(1,11)} = 59.55$; $P < 0.0001$], thus indicating the presence of learning in both genotypes. However, control mice acquired this task faster than TgDyrk1A, which showed significantly longer escape latencies in the first acquisition sessions (A1, A2; Fig. 4A). TgDyrk1A mice showed a significant increase in path length with respect to control mice on sessions 1 and 2 (A1, $t = 2.87$, $P = 0.015$; A2, $t = 2.76$, $P = 0.019$). In the cued non-hippocampal version of the task, both groups significantly differed, TgDyrk1A showing lower escape latencies than controls when spatial information was irrelevant ($t = 2.29$, $P = 0.043$). The session with the platform removed assessed the achievement of learning through the accuracy of mice in searching the previous location of the platform (Fig. 4A, central panel). In this task, TgDyrk1A mice were not effective in discriminating the previously trained quadrant whereas control mice showed an increased percentage of time spent navigating on the trained quadrant ($t = 2.51$; $P = 0.014$). In the reversal-learning test, the platform was hidden in the south-west quadrant, opposite to the trained one (north-east). In this task, transgenic animals showed a preference in searching in a non-trained (north-west) quadrant (Fig. 4A, right panel). Control mice showed no spatial bias for any quadrant, indicating a poor performance, although there was a tendency to present lower escape distances in this group (173 ± 47 in control mice versus 238 ± 33 in TgDyrk1A mice; $t = 0.6$, $P = 0.56$, not significant) (Fig. 4A, left panel).

To further assess spatial reference and working memory we used the repeated acquisition version of the Morris water maze task. In this more cognitively demanding version of the task, which implies a repeated reversal learning of platform position during 8 days, transgenic mice showed a significant increase in the average escape latencies and distance traveled in the first sessions (Fig. 4B, left panel). Escape distances in the repeated-reversal learning paradigm of the Morris water maze showed an increase in the distance traveled by the TgDyrk1A (L9) mice versus controls [increment 76.17, 95% confidence interval (CI) = 24.29,128.05; $t = 2.88$, $P = 0.0043$]. This impairment was specifically related to an alteration in reference memory (Fig. 4B, central panel) but not in working memory (Fig. 4B, right panel) since the differences were observed in the odd trials (increment 65.97, 95% CI = 5.84,126.10; $t = 2.15$, $P = 0.0401$), whereas the learning curve obtained in even trials showed no significant differences (increment 62.02, 95% CI = –13.42,137.46; $t = 1.61$, $P = 0.1091$), indicating specific alterations in the reference memory but not in working memory.

DISCUSSION

Transgenic mice overexpressing the full-length cDNA of *Dyrk1A* under the control of the inducible *sMT-Ia* promoter were viable, normal in their somatometric development and in the emergence of developmental landmarks, and showed no gross anatomical alterations.

Despite no alterations in the maturation of sensorial and reflexologic responses were observed, TgDyrk1A mice showed a persistence of immature locomotor patterns, accompanied by a delayed acquisition of mature locomotor activity. No previous report has specifically explored neuromotor development in other DS murine models, and therefore, our study provides the first evidence of the involvement of a HSA21 gene in this phenotypic aspect.

The most important delay was observed in a task evaluating the general psychomotor development, the homing test, where TgDyrk1A mice performed significantly worse than wild-type

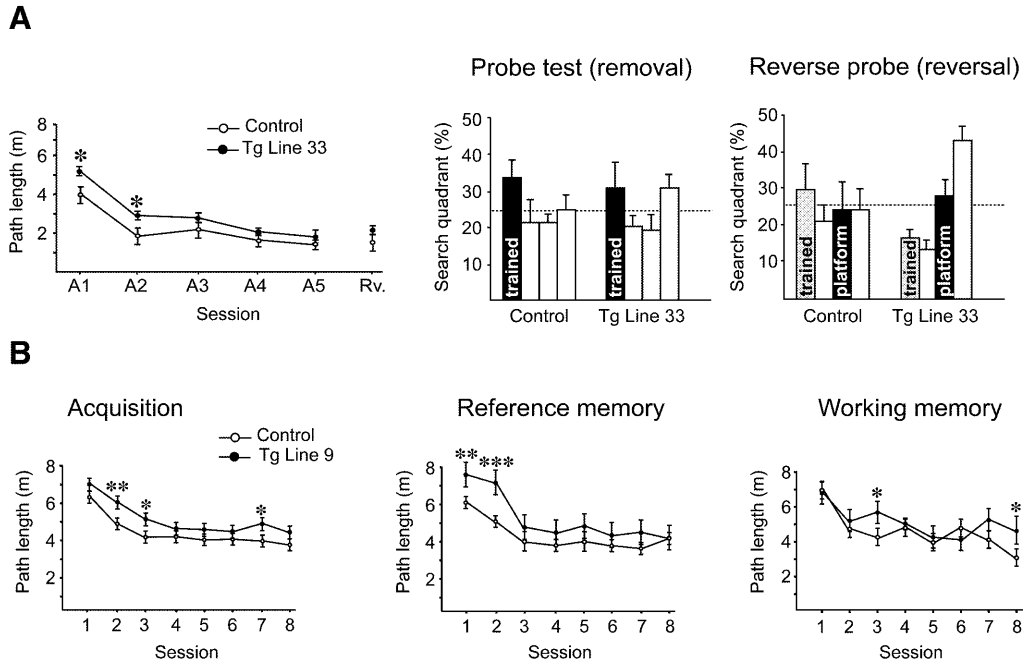

Figure 4. Performance in Morris water maze of TgDyrk1A and wild-type mice. (**A**) Repeated measures ANOVA revealed a significant effect of session on the escape distances in the hidden platform test both in wild-type and TgDyrk1A mice [$F_{(1,11)} = 59.55$, $P < 0.0001$; repeated measures ANOVA), thus indicating the presence of learning in both genotypes. TgDyrk1A (L33, $n = 6$, closed circles) showed a significant increase in path length with respect to control mice ($n = 7$, open circles) on sessions 1 and 2 (A1 and A2, left panel). In the removal test, whereas control mice showed an increased percentage of time searching the platform in the trained quadrant, TgDyrk1A did not exhibit this preference (central panel). In the reverse platform test, TgDyrk1A mice persisted in searching in a non-trained NW quadrant (right panel). However, the latency to arrive was similar in both groups (left panel). Data are expressed as mean ± SEM. $*P < 0.05$, $**P < 0.01$; repeated measures ANOVA with Bonferroni *post hoc* analysis. (**B**) Escape distances in the repeated-reversal learning paradigm of the Morris water maze showed an increase in the distance traveled by the TgDyrk1A (L9, $n = 11$, closed circles) versus control littermates ($n = 10$, open circles) (left panel). These differences are principally due to the worse performance in the odd trials, indicating specific alterations in the reference memory (central panel), but not in working memory (right panel). Data expressed as mean ± SEM. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

mice. The retardation in locomotor development observed in TgDyrk1A mice may have an influence on the homing of the pups to the nest during the suckling period. However, the homing test was performed at P14 when the retardation in walking activity was quite attenuated. Sensory abilities, which may also influence performance, were not affected by the overexpression of *Dyrk1A*. Notably, visual acuity and propioception were not altered. No specific test for olfactory ability was performed, and therefore olfactive impairment cannot be excluded to influence the poor performance on this test. Similar to our results Ts65Dn mice also show an altered response in the homing test (13,19) and a delay in sensorimotor development, reaching normal levels at weaning. In the present experiments, TgDyrk1A mice show a delay more specifically related to neuromotor development. This delay was only partially overcome with maturation since no impairment in gait was observed in the adult, but other tasks requiring adequate motor performance, such as the coat hanger test, remained markedly impaired. Taken together, our results suggest that overexpression of *Dyrk1A* produces a defect in motor activity probably related to motor coordination impairment or the presence of hypotonia, arising from neurodevelopment.

This motor alteration did not influence circadian activity in adult TgDyrk1A mice. Despite the absence of differences in the actimetry test, which provides an estimate of baseline activity in a familiar environment, increased locomotion in novel environments was consistently found along different test conditions. In the open field, TgDyrk1A mice traveled longer distances than control littermates, but only when the experiment was performed during the light phase of the photoperiod, in conditions where the level of anxiety generated by the test is higher. This hyperactivity was also exhibited in the elevated plus maze, as reflected by an increase in total activity. The increased activity was present in the protected and non-protected areas of both test arenas. TgDyrk1A mice showed an increase in the number of crossings in the center of the arena in the open field and entered more often, traveled longer distances and spent more time in the open arms in the plus maze, parameters considered to be a measure of anxiety in rodents. Taken together, these experiments demonstrate that TgDyrk1A mice are hyperactive in situations that usually provoke caution in normal mice, a result that can be interpreted as a failure of TgDyrk1A mice to inhibit activity. Similar to our findings, Ts65Dn mice are also unable to inhibit exploration of the open arms in the plus maze (20). This inability to inhibit behavior has been ascribed to dysfunction of prefrontal cortex, specifically related to hyperactivity in rodents (21). Patients with DS are more likely to be hyperactive than normal children (22) but the prefrontal deficit is more markedly expressed as a deficient behavioral inhibiting function (23). Specific overexpression of *Dyrk1A* leads to alterations in locomotion and behavior, which could explain to some extent the behavioral alteration of DS patients. Our results are in contradiction with those of the transgenic TgYAC157Ftel mice, which were

described as hypoactive (16). However, this hypoactivity was very slight and only observed in non-stressful conditions (dimly illuminated arena), in which no significant hyperactivity was observed in TgDyrk1A. Our results suggest that the overexpression of *Dyrk1A* may have fairly specific, although subtle, long term consequences on activity and behavior.

Is the overexpression of *Dyrk1A* associated with memory alterations? To investigate the cognitive alterations in TgDyrk1A mice, the hippocampally mediated memory performance was assessed in the Morris water maze. The first experiment, using a spatial reference memory procedure, showed that during acquisition both control and transgenic mice learned to reach the platform. However, TgDyrk1A mice experienced more difficulties in learning the task as shown by the significantly longer escape latencies in the first acquisition sessions. In the cued non-hippocampal version of the task, both groups significantly differed, TgDyrk1A showing lower escape latencies than controls when spatial information was irrelevant. These results indicate that TgDyrk1A mice were either more motivated to escape from the water or more capable of orienting themselves using intra-maze cues. Other DS murine models such as Ts1Cje (15), trisomic for the segment of the distal region of MMU16, or the transgenic YAC152F7tel mice (16), performed efficiently in the Morris water maze when the platform was visible. However, Ts65Dn mice, with a larger MMU16 trisomic segment, show significant deficits in the visible platform component of the task (13).

The removal test assessed the achievement of learning through the accuracy of mice in searching the previous location of the platform. In this task, control mice were significantly more effective in discriminating the previously trained quadrant, as shown by the increased percentage of time spent navigating on the previous platform location. On the contrary, TgDyrk1A mice did not show any spatial bias towards the trained quadrant, displaying equal preference in searching time for either the trained and the adjacent quadrants. In the probe test, all murine models encompassing *Dyrk1A* have shown different degrees of impairment. While YAC152F7tel mice showed only mild impairment in the hidden platform and probe tests (16), Ts65Dn and Ts1Cje mice are more importantly impaired (13–15,22,24).

In the reverse platform test, the mice are required to learn the novel position of the hidden platform, located on the opposite quadrant. In this phase, TgDyrk1A mice were poor in learning the new location of the platform and traveled longer distances to reach the platform in the new position. Moreover, they persisted in searching in the wrong quadrant, indicating a poor cognitive flexibility and a random strategy. Cognitive flexibility requires that the animal does not persevere in choosing the wrong solution, and has been ascribed to the correct functioning of the prefrontal cortex. Thus, the fact that reversal and probe learning are more impaired than initial discrimination learning in TgDyrk1A suggests that forebrain structures underlying behavioral flexibility, but also probably hippocampal structures, could be impaired. Similar results in the reverse platform test have been obtained in YAC152F7tel transgenic mice (16) and in the partial MMU16 trisomy models (24). Thus, imbalance of the *DYRK1A* region could be important in determining the alteration of forebrain regions, with an impact in cognitive function. The specific contribution of *Dyrk1A* to the partial MMU16 model phenotype and probably to DS is highlighted by the deficit in the development of learning strategies observed in TgDyrk1A mice.

To further assess spatial reference and working memory, we used the repeated acquisition version of the Morris water maze task. In this more cognitively demanding version of the task, which implies a repeated reversal learning of platform position during 8 days, transgenic mice showed a significant increase in the average escape latencies and distance traveled in the first sessions. This impairment was specifically related to an alteration in reference memory but not in working memory. These alterations are comparable with those detected in the Ts65Dn mice (14) and clearly extend the findings described for the YAC152F7tel mice (16). TgDyrk1A mice are more severely impaired than YAC152F7tel mice. This could be produced by the fact that the TgDyrk1A construct only contains *Dyrk1A* cDNA, whereas more genes have been shown to be included in the YAC used for generating YAC152F7tel mice (J.Delabar, personal communication). It might be argued that the heterologous promoter used for TgDyrk1A construction could drive the expression of Dyrk1A in a different pattern of endogenous Dyrk1A. However, expression studies showed a similar pattern of expression between controls and TgDyrk1A mice. Alternatively, differences in the genetic backgrounds and experimental environments or procedures could also affect the performance in the behavioral tests (25). However, it should be pointed out that TgDyrk1A impairment was detected using two different learning paradigms and in two different transgenic lines, strongly supporting the existence of a visuo-spatial learning defect in TgDyrk1A mice.

DYRK1A has been proposed as a candidate gene implicated in DS phenotype on the basis of its location in the DS critical region, its overexpression in DS fetal brain and its putative role in neuronal proliferation. The present experiments demonstrate that overexpression of *Dyrk1A* in mice produces a specific retardation in neuromotor development and hyperactivity in the adult that seems to be dependent on environmental factors, mainly the presence of stressors. Cognitive deficits, including spatial learning that requires the integration of visual and spatial information, without alterations in working memory, are consistently present in TgDyrk1A mice, and reveal a profile that is indicative of hippocampal and prefrontal dysfunction. In conclusion, our findings clearly indicate that *DYRK1A* overexpression could be sufficient to cause learning deficits and neurodevelopmental alterations in DS patients.

MATERIALS AND METHODS

Animals

Experiments were carried out in C57BL6/SJL mice. They were housed in standard macrolon cages with freely available food and water. Standard environmental conditions of humidity and temperature (22 ± 1°C) were kept constant and a 12 h light/dark (LD) cycle was used (6:00 a.m. to 6:00 p.m.). All the procedures were performed in accordance with recommendations for the proper care and use of laboratory animals.

Construction of *sMT-Ia/Dyrk1A* transgene

The *Eco*RI/*Xho*I fragment containing the *sMT-Ia* promoter was introduced at the *Eco*RI/*Xho*I site of the pCMVβ plasmid and was designated psMT. The full-length *Dyrk1A* cDNA (a gift from Dr W.Becker, Hamburg, Germany) was PCR amplified to introduce *Not*I sites at the 5′ and 3′ ends and was then cloned at the *Not*I site of the psMT-Ia plasmid. The complete transgene sequence was subsequently confirmed by DNA sequencing.

Generation of transgenic mice and genotyping

Transgenic mice have been generated by standard microinjection procedures. Fertilized mouse eggs were flushed out from the oviducts of superovulated C57BL6/SJL mice. Male pronuclei of the fertilized eggs were injected with the DNA solution (containing the 6.7 kb *Avr*II–*Sal*I fragment from the *sMT-Ia/Dyrk1A* chimeric gene) and viable embryos were reimplanted in the oviducts of pseudopregnant mice. At 3 weeks of age, the animals were tested for the presence of the transgene by Southern blot analysis from tail samples. Ten micrograms of DNA was digested with *Pst*I, electrophoresed in 1% agarose and transferred to nitrocellulose membranes (Hybond-N$^+$; Amersham Pharmacia Biotech). Hybridization of the filters was performed according to the manufacturer's procedures (Amersham Pharmacia Biotech), using a 1.5 kb *Pst*I fragment of the transgene as a probe. Four transgenic lines were obtained and maintained in hemizygosity by crossing with C57BL6/SJL mice. Transgene copy number was also determined by Southern blot analysis. Autoradiographs were analyzed using Phoretix 1D software (Nonlinear Dynamics Ltd). Genotyping was performed routinely by PCR analysis using the primer pair: DYRKf primer, 5′-GTC CAA ACT CAT CAA TGT ATC-3′ and DYRKr primer, 5′-CTT GAG CAC AGC ACT GTT G-3′. Each cycle (32 cycles) consisted of 94°C for 30 s, 52°C for 30 s and 72°C for 45 s.

Expression analysis of the transgene

Total RNA from various tissues was isolated with the TriPure kit (Boehringer Mannheim) and analyzed by RT–PCR and northern blot. RT–PCR was carried out by reverse-transcribing total RNAs (1 µg) using Superscript reverse transcriptase (Gibco BRL). The cDNA solution was subjected to 40 cycles of PCR amplification in the same conditions described above for genotyping with the primers (DYRKf and DYRKr). Absence of genomic DNA contamination was determined by the amplification of a 126 bp PCR fragment from cDNA samples with primers for GdX transcript (GdXf, 5′-GGC AGC TGA TCT CCA AAG TCC TGG-3′; GdXr, 5′-AAC GTT CGA TGT CAT CCA GTG TTA-3′).

Equal amounts of total RNA (20 µg per lane) were electrophoresed on formaldehyde-containing 1% agarose gel, and subjected to northern transfer on a nitrocellulose membrane. Filters were hybridized following standard protocols and using a ^{32}P-labeled 1.5 kb cDNA fragment as a probe that recognizes both the endogenous and the transgene transcripts. The levels of mRNA were determined by densitometric scanning of autoradiograms. Densitometry values were corrected by using ribosomal ethidium bromide signal as internal standard. Western blot analysis from adult whole brain was performed as described by Okui *et al.* (26). Fifty micrograms of proteins were resolved on 7.5% SDS–PAGE (Bio-Rad MiniProtean II Cell system). Proteins were electro-blotted onto membranes (Hybond-C, Amersham Pharmacia Biotech). Membranes were blocked and incubated overnight in a solution containing 1:100 anti-Dyrk monoclonal antibody (Transduction Laboratories, D57220). Incubation with anti-mouse IgG/HRP followed by enhanced chemiluminescence (ECL) assay allowed detection. Actin was used as internal standard, using anti-Actin antibody (Sigma, A-2066). Protein concentration was determined by a bicinchinonic acid (BCA) assay (Pierce).

Histological analysis

Mice were perfused transcardially with phosphate-buffered saline (PBS) and then with 4% paraformaldehyde in phosphate buffer (PB). The brains were removed from the skull and left in the same fixative for 24 h. Brains were then embedded in paraffin or cryoprotected and coronal sections were obtained using a sliding microtome (5 µm) or a cryostat (30 µm). Tissue sections were subjected to H&E staining or processed for immunohistochemistry with anti-Dyrk antibody. The sections were processed free-floating using the streptavidin-biotin-peroxidase complex immunohistochemical method (DAKO, LSAB system, peroxidase). Briefly, after peroxidase blocking, sections were incubated with 10% fetal bovine serum and 0.25% gelatin. Then incubation with the primary antibody was performed overnight at 4°C at a dilution of 1:50 in PBS containing 0.2% Triton X-100 and 1% fetal bovine serum. The sections were then incubated with the biotinylated link and the streptavidin-HRP, 10 min each, as indicated in the manufacturer's instructions. Peroxidase activity was visualized with 0.05% diaminobenzidine and 0.01% hydrogen peroxide.

Behavioral analysis

TgDyrk1A (lines L9 and L33) and wild-type littermates F1 from eight different litters were used for the developmental analysis (from P1 to P21). For the behavioral studies in the adult male TgDyrk1A (lines L9 and L33) mice of 4 months of age were used. All the behavioral studies were performed for both lines under basal (non-induced) conditions. Studies in the adult were performed in the same animals that were submitted to the neurodevelopmental test battery.

Developmental studies

All the pregnant mice were allowed to deliver spontaneously. The day of delivery was designated as day 1 of the neonates. On the morning of delivery the litter size was recorded and each pup was checked for gross abnormalities. The pups were nursed by their natural dams until weaning and were weighed every day. The developmental screening used males and females of the different genotypes. The test includes sensorial and motor responses based on the method developed by Fox (27), with some modifications.

The pups from the different litters were weighed daily to the nearest 0.01 g. Body and tail length were measured to the nearest 1 mm. Pups were observed for pinna detachment, development of fur, incisor teeth eruption, permeation of the ear canal and eye opening. The presence of a startle response and the surface righting reflex were also evaluated. Sensorimotor

parameters were evaluated, including prehensile reflex, visual acuity and aversion to fall. These reflexologic and behavioral tests reflect the maturation of the CNS, are reactive to environmental and toxic conditions and their reliability is high.

Preweaning motor behavior

Neuromotor development was assessed on P7, P10 and P14 by means of the pivoting and walking tests. Pivoting locomotion test was performed on a flat surface with lines drawn to delineate four 90° quadrants. The total number of degrees turned by the pup only in completed 90° segments, during 60 s was recorded. In the walking test the latency for a mouse to lift up on all four legs and walk a distance exceeding its body length was measured. For the homing test, P14 pups were individually transferred to a cage containing new sawdust in two-thirds and sawdust of the home litter ('goal arena') in the distal third. The pups were placed in the opposite side of the goal arena, near to the wall. The latency to reach the home litter sawdust was recorded (cut-off time 180 s). In the wire suspension test pups were placed on a wire (4 mm diameter) in an upside-down position. The ability of the animal to remain suspended was measured as the latency to fall.

Motor coordination

In adult mice motor coordination was measured using a coat-hanger test; the mice were placed in the middle of the wire in an upside-down position. The latency until falling down and the activity performed on the wire were measured in one trial over 60 s.

Open field test

The open field was a white wooden box ($70 \times 70 \times 25$ cm high) divided into 25 equal squares. The number of squares crossed with four paws, the distance traveled (in the center and the periphery), rearing activity, defecation (number of foecal boluses) and grooming behavior were scored during 5 min. The test was conducted during the dark phase of the LD cycle under red light (20 luxes) and during the light phase of the cycle (1500 luxes).

Elevated plus maze

The mice were tested in the elevated plus maze (5 min) during the dark phase of the LD cycle under red light (20 lux). The plus maze was made of black plexiglas and consisted of two open (30×5 cm) and two enclosed arms ($30 \times 5 \times 15$ cm) with a 5 cm square center, elevated 40 cm above the floor. The number of entries into arms, the distance traveled and the time spent in each arm were tracked during 5 min by a video camera connected to a video-tracking system (SMART, Letica-Panlab).

Water maze task

Two different learning paradigms based on the Morris water maze task were performed. Mice from line 33 were tested for place learning acquisition in the water maze pool over 5 days (four trials per session). The platform was placed in a fixed position in the center of the north-east quadrant, 1 cm below the water surface. In each trial, mice were placed at one of the starting locations in random order [north, south, east, west (N, S, E, W), including permutations of the four starting points per session] of a swimming pool (120 cm diameter, depth 25 cm) filled with water (24°C) made opaque with milk. The animals were allowed to swim until they located a platform (15 cm diameter, height 24 cm) submerged in a fixed position (SW quadrant, 22 cm away from the wall). Mice failing to find the platform within 60 s were placed on it for 30 s (the same period of time as the successful animals). Several fixed room cues were constantly visible from the pool. At the end of every trial the mice were allowed to dry for 15 min in a heated enclosure and returned to their home cage. In the cued learning session, the platform was elevated 1 cm above the water and its position was clearly indicated by a visible cue. White curtains prevented the use of extra-maze cues. In the probe session [four trials entering the pool from the four different starting points (N, S, E and W)] the platform was removed and mice were allowed to swim for 60 s without platform. The time spent in the trained and non-trained quadrants as well as the number of platform annulus crossings during 60 s were recorded. On the next day, mice performed the reversal learning session. In this test, the platform position was changed to the opposite quadrant (SW). If mice were unable to find the platform within 60 s they were placed on it for 30 s.

The repeated acquisition protocol was performed with mice from line 9 as described previously (14). Briefly, the experiment consisted of eight acquisition sessions and four cued sessions, each consisting of four pairs of trials. From one daily session to the next, the platform was placed in a different location (N, S, E, W and center). Mice randomly started from each of the four positions on the first (even) trial of a pair and from the same starting position in the second (odd) trial of the pair. The first trial of a pair was terminated when the mouse located the platform or when 60 s had elapsed; following a period of 20 s in which the animal was allowed to stay on the platform, the second trial of the pair was run immediately.

Escape latencies, path lengths and swimming speed for each mouse and trial were provided by a tracking system (Smart, Letica S.A.) connected to a video camera placed above the pool.

Data analysis

When no significant differences were detected from transgenic mice of both lines, results were combined. Unless stated otherwise, significance of the effects was assessed by a one-way ANOVA or multivariate analysis of variance (MANOVA) with Bonferroni test for *post hoc* analyses. Student's *t*-test was used for comparisons between two groups. Repeated measures ANOVA was used for pair-wise comparisons of two groups. In the repeated reversal-learning paradigm in the Morris water maze, and due to the unbalanced design, general linear-mixed models were used to estimate the effect of genotype on the dependent variables. The general linear-mixed models allowed testing of the overall effect, at any acquisition session (from S1 to S8) and any group (control and TgDyrk1A mice), the session effect, which measured differences at the 2nd to 8th sessions with respect to the 1st session, and the interaction between group and session, which measured different effects at different sessions. In order to check the models we plotted the residuals versus fitted values. The variance function structure

was used to model heteroscedasticity in the within-group errors. All models included subject as random effect. The test for the need of a random effect for genotype was not statistically significant. Estimation of coefficients and standard errors to compute 95% confidence intervals were based on restricted maximum likelihood. Comparisons of models were based on likelihood ratio tests derived from model fits using maximum likelihood fit. Expected values derived from the simplest model fitted to the data were used to plot the effect of dependent variables in relation to group and session of evaluation. All analyses were performed with S-PLUS functions using the library *nlme* (28).

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