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Hyperactivity and Enhanced Curiosity of Mice Expressing PKB/SGK-resistant Glycogen Synthase Kinase-3 (GSK-3)

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Key Words

Light-dark box • O-maze • Locomotion • Mania • Behavior • GSK3 • Depression • Bipolar • Forced swimming test

Abstract

Background: Mounting evidence suggests that bipolar disorder symptoms could be favorably influenced by modification of glycogen synthase kinase-3 (GSK-3) activity. Specifically, the well known antimanic and mood stabilizing medications lithium, valproate, olanzapine and clozapine have been shown to inhibit GSK-3 activity. GSK-3 is phosphorylated and thus inhibited by protein kinase B (PKB/Akt) and serum and glucocorticoid inducible kinase (SGK) isoforms. The present study explored, whether PKB/SGK-dependent GSK-3 regulation influences the behavior of mice. Methods: Gene-targeted knockin mice with mutated and thus PKB/SGK-resistant GSK-3α,β (*gsk-3*^{KI}) were compared to corresponding wild type mice (*gsk-3*^{WT}). The mice were analyzed by open-field, light-dark (LD-) box, O-maze, emergence test, object exploration test and forced swimming test (FST). Results: In open-field, LD-box and O-maze, *gsk-3*^{KI} mice displayed a hyperactive and more curious phenotype when compared to wild type mice. Speed and total distance moved as well as rearings were significantly increased in *gsk-3*^{KI} compared to *gsk-3*^{WT} mice. In the O-maze, *gsk-3*^{KI} mice tended to travel a larger distance in the open, unprotected area than *gsk-3*^{WT} mice, and performed significantly more unprotected head dips suggesting decreased anxiety behavior. In the forced swimming test, the immobility time was significantly decreased in *gsk-3*^{KI} mice indicating a phenotype less prone to depression. Moreover, *gsk-3*^{KI} mice were less sensitive to the application of chronic mild stress and showed a decreased HPA axis activity. Conclusions: The present observations disclose a significant role of PKB/SGK-dependent regulation of GSK-3 in the control of activity, anxiety and proneness to depression. Accordingly, mice expressing SGK/PKB resistant GSK-3 may be a valuable model of hyperactivity and mania.

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Introduction

Mood disorders are common disorders and include major depression and bipolar disorders. They are widely distributed with a life prevalence of up to 20% [1]. Usually depressive symptoms are associated with substantial symptom severity, role impairment and enormous costs of lost productive work time [2]. While the recent increase in treatment is encouraging, inadequate treatment is a serious concern. Antidepressant treatment involves amelioration of symptoms over the course of weeks or months and remains uneffective in many patients.

However, recent evidence points to a common downstream target of several antidepressant and mood stabilizing treatment strategies. Lithium, valproate, olanzapine and clozapine all activate PKB and lead to glycogen synthase kinase (GSK-3) phosphorylation [3-5]. GSK-3 is phosphorylated and thus inhibited by PKB [6, 7] and SGK [8, 9], kinases activated through the phosphatidylinositol 3 kinase (PI3-K) pathway [10]. The resulting inhibition of GSK-3 has been considered to account for the beneficial effects of mood stabilizing drugs in bipolar disorders [3, 4]. Moreover this inhibition of GSK-3 has been shown to yield antidepressant-like effects [11-13]. Along those lines PI3-K is activated by brain-derived neurotrophic factor (BDNF) [14], which has been implicated mainly in the pathophysiology and treatment of depression and bipolar disorder [15-18]. The PI3-K pathway has further been implicated in the regulation of behavior and especially in schizophrenia [17, 19-21].

Additionally other antidepressant treatment strategies activate the PI3-K/PKB pathway, i.e. estrogen, physical exercise and electroconvulsive therapy (for review see [22]), and the stress hormone dexamethasone inhibits the PI3-K cascade . As shown recently [24], transgenic mice overexpressing GSK-3 display strikingly increased general locomotor activity, decreased habituation in an open-field, increased acoustic-startle response, and reduced immobility in the forced swimming test.

GSK-3 is regulated by several signaling pathways, including those activated by Wnts, hedgehog, growth factors, cytokines, and G-protein-coupled ligands [25]. GSK-3β could be activated by phosphorylation at the tyrosine 126 [26] and GSK-3 could be inhibited by phosphorylation at serines 21 (GSK-3α) or 9 (GSK-3β) [27]. Replacement of serine within the PKB phosphorylation site by alanine (GSK-$3\alpha^{21A/21A}$, GSK-$3\beta^{9A/9A}$) renders GSK-3 resistant to inactivation by PKB/SGK [27]. Accordingly, the effect of insulin on muscle glycogen synthase is abrogated in knockin mice carrying these mutations *(gsk-3^{KI})* [27]. On the other hand, Wnt-dependent signaling of GSK-3 signaling is not impaired in those mice [27].

The present study explored, whether PKB/SGK-dependent inhibition of GSK-3 participates in the regulation of behavior. To this end, several behavioral experiments have been performed in *gsk-3^{KI}* mice and corresponding wild type animals (*gsk-3^{WT}*).

Materials and Methods

All animal experiments were conducted according to the guidelines of the American Physiological Society as well as the German law for the welfare of animals, and were approved by local authorities.

Mice were generated, in which the codon encoding the Ser9 of the GSK-3β gene was changed to encode nonphosphorylatable alanine (GSK-$3\beta^{9A/9A}$), and simultaneously the codon encoding the Ser21 of the GSK-3α gene was changed to encode the nonphosphorylatable GSK-$3\alpha^{21A/21A}$ thus yielding the GSK-$3\alpha/\beta^{21A/21A/9A/9A}$ double knockin mouse (*gsk-3^{KI}*) as described previously [27]. Mice had a mixed Sv129 and C57Bl6 background.

Mice were compared to corresponding age and sex matched wild type mice (*gsk-3^{WT}*). GSK-$3\alpha/\beta^{21A/21A/9A/9A}$ double knockin mice (*gsk-3^{KI}*) and wild type mice (*gsk-3^{WT}*) were obtained from homozygote breeding. To avoid fighting of males, mice were housed alone or in groups of maximal 3 mice during the experimental period. 11 *gsk-3^{WT}* and 11 *gsk-3^{KI}* mice for both the control and the stress experiments were used. The age of the animals was 3.8-5.0 or 1.9-3.0 months at the beginning and 5.0-6.2 or 2.9-3.9 months, respectively, at the end of the experiments. They were fed a control diet (1310, Altromin, Lage, Germany) and had free access to tap water. Two months before the experimental period animals were maintained at a 12:12 h inverted cycle with lights on between 7 p.m. and 7 a.m. Standard mouse chow, water and nesting material were available ad libitum. Behavioral testing occurred between 7 a.m. and 7 p.m. Only one type of experiment was done on the same day and the home cage was brought to the test room at least 30 min before each experiment started. Dry surfaces of the apparatus were thoroughly cleaned with 70% ethanol and water before releasing the animal. Experiments extended over a total of 5.9 or 4.7 weeks.

Application of chronic mild stress (CMS) started 3 weeks before the first experiment. The different stress factors were selected according to the protocol of Willner [28, 29].

For data acquisition animals were video tracked by the camera 302050-SW-KIT-CAM at a resolution of 0.62 to 0.72 pixel (TSE-Systems, Germany, www.TSE-Systems.com). Raw data were transferred to Excel for further analysis.

Tests were done in the following order: open-field, LD-box, O-maze, emergence test, object exploration test and forced swimming test. Experiments were performed with diffuse indirect room light produced by dimmable bulbs, adjusted to

yield less than 30 lux in the center of the experimental arena. The only exception was the LD-box test where full room light was switched on.

The open-field is a method to analyze locomotor and mood-related behavior. Speed and total distance moved provide information about the general activity of mice. Speed was analyzed as distance per time whereas the distance between two positions of the barycentre of the mouse is calculated on the basis of the Pythagoras theorem. All distances were then added up to the total distance. Furthermore, it is a matter of particular interest which zone of the open-field the mice prefer. Anxious behavior is reflected by staying in the corners and the border area, whereas audacious behavior is indicated by exploration of the center of the arena. The quadratic arena had side lengths of 50 cm, a white plastic floor, and 40 cm high sidewalls. Each subject was released near the wall and observed for 30 min [30]. For evaluation, a border with 10 cm distance to the wall was considered.

The light-dark (LD-) box is based on the fact that mice avoid bright light but at the same time want to explore unknown environment. The LD-box creates a conflict for the mouse that tends to explore the unfamiliar area and initially wants to avoid the unfamiliar (neophobia). The fraction of time spent in the box is seen in correlation to anxiety and depression. An infra-red permeable box made of black acryl (40 cm high) was inserted into the open-field and covered 33% of the surface area. An aperture of 10 cm length and 11 cm height with rounded down corners led from the light arena to the dark box. The experiment was performed under direct room light. Each subject was released in the corner of the lit compartment and observed for 5 min [31].

The O-maze is an additional model to investigate anxiety-related behavior. The experiment tests the urge of the mice to discover and their instinct to protect themselves. Mice usually avoid dangerous areas, but override their anxiety after a certain time because of curiosity. Important measures are for example if and how quickly they dare to leave the original protecting area and enter the opposing area. A 5.5 cm wide annular runway was constructed using white polyvinyl-chloride. It had an outer diameter of 46 cm and was placed 40 cm above the floor [21, 32]. The two opposing 90° closed sectors were protected by 11 cm high inner and outer walls of white polyvinyl-chloride, while the remaining two open sectors had a border of 5 mm. Animals were released in one of the closed sectors and observed for 10 min. A diagram illustrates the experimental set-up in Fig 1.

For the emergence test, the procedure was modified after Dulawa et al. [33]. An infra-red permeable box, which was previously in the home cage of the mouse during more than 24 hours, was inserted into the arena. The home box had a base area of 9x10 cm with two apertures (3 cm length, 3.5 cm height with rounded down corners) on the long sides. The mouse was observed for 30 min. This test examines the behavior of the mice in an area during the presence of a familiar object. The main interest is the time spent in or near the familiar home box.

For the object exploration test, the procedure was modified after Dulawa et al. [33]. In addition to the familiar home box there is a novel object put into the area to investigate the exploratory behavior of the mouse in a partially familiar environment. The mouse was first observed for 30 min in the open-field including the home box. Afterwards, a falcon tube was additionally put into the area and fixed with adhesive tape. The mouse was then observed for another 30 min and the contact with the nose on the falcon tube was taken as criterion to analyze the reaction on a novel object.

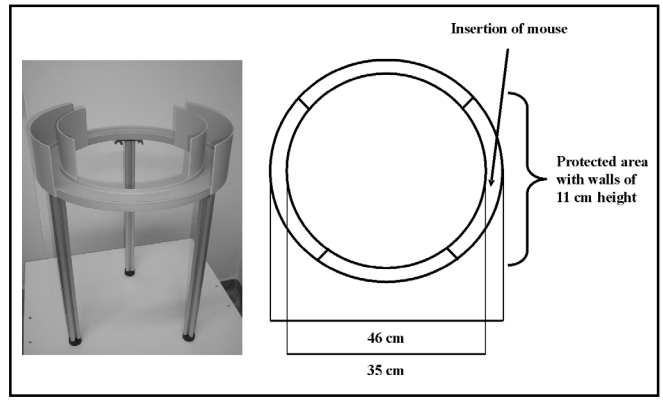

Fig. 1. Diagram of the O-maze.

In the forced swimming test mice where placed in a glass of water with temperatures between 24 and 26 °C. The diameter of the glass represented 20 cm, and the mouse was placed in the water without being able to touch the ground. Mice were observed during 6 min and the time they spent without movement, called floating, was recorded. It has been shown that the time of immobility is reduced by antidepressive drugs [24, 34]. Thus differences between genotypes may allow conclusions about their depressive behavior.

Data are provided as means ± SEM, n represents the number of independent experiments. All data were tested for significance using 1-way ANOVA analysis and the repeated measures ANOVA as appropriate. Only results with $P < 0.05$ were considered statistically significant.

Results

In the open-field, both speed (Fig. 2A) and total distance traveled (Fig. 2B) were significantly increased in *gsk-3^{KI}* mice when compared to *gsk-3^{WT}* mice whereas the habituation was similar. Stress reduced both measures significantly (Fig. 2C and 2D) - in the *gsk-3^{WT}* during the first 20 min, in *gsk-3^{KI}*, who started with a higher speed, from 10 min on. Furthermore, the decrease of speed during the second third of the experiment was more pronounced in *gsk-3^{KI}* than in *gsk-3^{WT}* mice, pointing to more rapid habituation. Moreover, the duration (Fig. 3A) and number (Fig. 3B) of rearings at the border of the open-field were significantly larger in

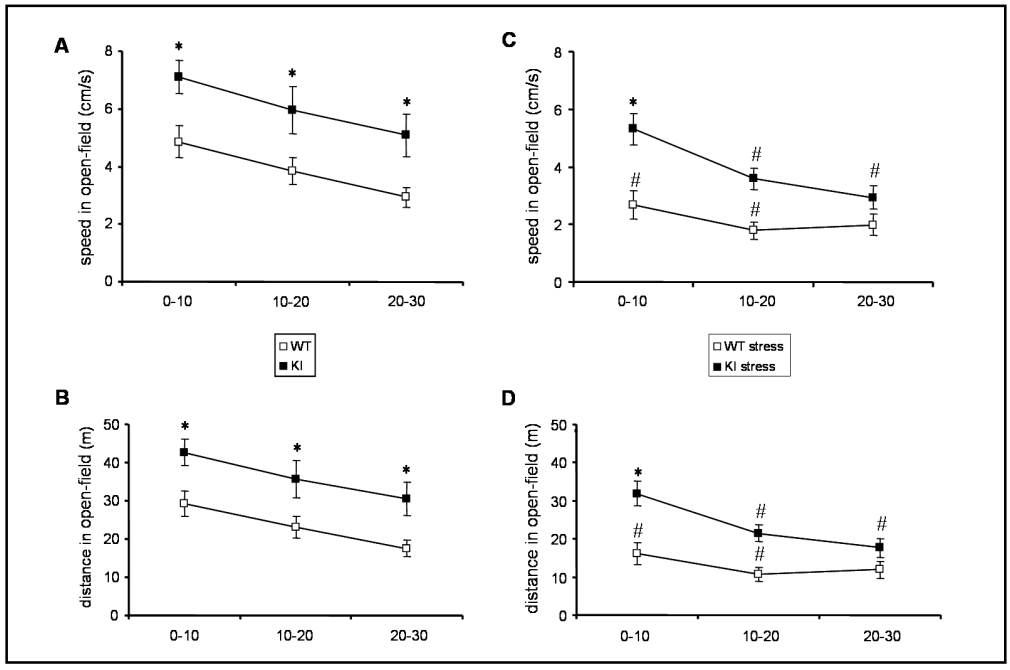

Fig. 2. Speed and distance traveled of *gsk-3*^{KI} and *gsk-3*^{WT} mice in the open-field. A. Arithmetic means ± SEM (N = 10-11 each group) of speed under normal conditions in the open-field during the first, second and third 10 minutes. *Gsk-3*^{WT} (WT): 4.86 ± 0.55 cm/s, 3.83 ± 0.47 cm/s, 2.93 ± 0.35 cm/s vs. *gsk-3*^{KI} (KI): 7.11 ± 0.57 cm/s, 5.95 ± 0.82 cm/s, 5.08 ± 0.74 cm/s. B. Arithmetic means ± SEM (N = 10-11 each group) of distance under normal conditions traveled in the open-field during the first, second and third 10 minutes. WT: 29.12 ± 3.32 m, 23.01 ± 2.85 m, 17.55 ± 2.09 m, KI: 42.69 ± 3.42 m, 35.68 ± 4.92 m, 30.46 ± 4.43 m. C. Arithmetic means ± SEM (N = 11 each group) of speed under stressed conditions in the open-field during the first, second and third 10 minutes. WT: 2.68 ± 0.48 cm/s, 1.78 ± 0.29 cm/s, 1.99 ± 0.36 cm/s, KI: 5.31 ± 0.55 cm/s, 3.58 ± 0.37 cm/s, 2.94 ± 0.41cm/s. D. Arithmetic means ± SEM (N = 11 each group) of distance under stressed conditions traveled in the open-field during the first, second and third 10 minutes. WT: 16.08 ± 2.91 m, 10.7 ± 1.74 m, 11.93 ± 2.19 m, KI: 31.87 ± 3.29 m , 21.48 ± 2.24 m, 17.65 ± 2.47 m. * indicates a significant difference between genotypes (1-way ANOVA, $p < 0.05$). # indicates a significant difference between treatment with stress (1-way ANOVA, $p < 0.05$).

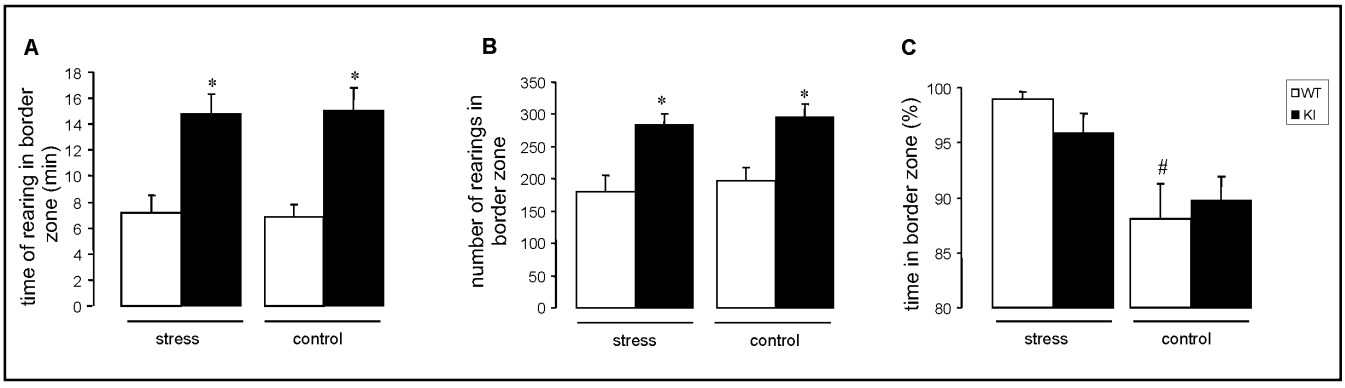

Fig. 3. Performance of *gsk-3*^{KI} and *gsk-3*^{WT} mice in the open-field. A. Arithmetic means ± SEM (N = 10-11 each group) of time of rearing in the border zone of the open-field. WT stress: 7.16 ± 1.38 min, KI stress: 14.78 ± 1.54 min, WT: 6.87 ± 0.92 min, KI: 15.02 ± 1.79 min. B. Arithmetic means ± SEM (N = 10-11 each group) of number of rearings in the border zone of the open-field. WT stress: 179.27 ± 26.05, KI stress: 282.91 ± 17.58, WT: 197.27 ± 20.86, KI: 296.2 ± 19.98. C. Arithmetic means ± SEM (N = 10-11 each group) of time spent in the border zone of the open-field. WT stress: 99.0 ± 0.64 %, KI stress: 95.83 ± 1.87 %, WT: 88.03 ± 3.2 %, KI: 89.81 ± 2.11 %. * indicates a significant difference between genotypes (1-way ANOVA, $p < 0.05$). # indicates a significant difference between treatment with stress (1-way ANOVA, $p < 0.05$).

gsk-3^{KI} mice than in *gsk-3*^{WT} mice, irrespective of stress. The fraction of time spent within the border zone was similar in *gsk-3*^{KI} mice and *gsk-3*^{WT} mice (Fig. 3C) whereas the distance moved in that area was significantly elevated in *gsk-3*^{KI} (Tab. 1). Under stress, both groups spent more time in the border zone. This effect was

Behavioral element	*gsk-3^{WT} stress*	*gsk-3^{KI} stress*	*gsk-3^{WT}*	*gsk-3^{KI}*	Statistics
Open-field					
Distance in border area (m)	37.5±5.9	65.8±5.8	54.9±4.6	90.8 ±8.4	WT vs. KI: p<0.01; KI vs. KIstress: p<0.05 WTstress vs. KIstress: p<0.05
Number of rearings in center	5.8±3.8	22.1±9.3	35.3±12.6	50.1±13.4	
Time of rearings in center (min)	0.22±0.15	1.04±0.5	1.39±0.55	1.88±0.57	
Mice performing rearing in center at all	3/11=27.3%	8/11=72.7%	10/11=90.9%	10/10=100%	
Number of visits of the center	8.82±5.2	35.0±12.7	78.0±17.9	94.0±17.5	WT vs. WTstress: p<0.01; KI vs. Kistress: p<0.05
Mice visiting the center at all	6/11=54.6%	8/11=72.7%	10/11=90.9%	10/10=100%	
Distance in center (m)	1.15±0.68	5.14±1.96	14.81±3.9	18.08±3.98	WT vs. Wtstress: p<0.05 KI vs. Kistress: p<0.05
Light-dark box					
Ratio time / no. of rearing in box			1.43±0.04	1.9±0.08	WT vs. WT stress: p<0.01; WTstress vs. KIstress: p<0.05
Ratio time / no. of rearing in light			1.44±0.15	1.90 ± 0.21	
Distance moved [m]	2.73±0.39	2.99±0.7	2.04±0.5	4.48±0.65	WT vs. KI: p<0.05
O-maze					
Distance traveled [m]	21.6±1.94	32.9±2.75	15.0±1.32	19.7±1.31	WT vs. KI: p<0.01; KI vs. KIstress: p<0.001
Speed [cm/s]	3.60±0.32	5.48±0.46	2.5±0.22	3.28±0.22	WTstress vs. KIstress: p<0.01 KI vs. KIstress: p<0.001
Body weight at the beginning of experiments with stress	26.03±1.15	30.18±0.78			WTstress vs. KIstress: p<0.01
Body weight at the end of experiments with stress	27.63±1.04	32.03±0.66			WTstress vs. KIstress: p<0.01
Emergence Test					
Time spent in home box [%]			60.9±8.32	64.3±6.63	n.s.
Distance moved in home area [m]			26.1±1.51	30.5±1.59	n.s.
Visits in box			84.0±9.26	109.2±14.8	n.s.
Object exploration test					
Time spent in home box during first 30 min (without object) [%]			53.4±8.7	69.4±7.0	n.s.
Time spent in home box during second 30 min (with object) [%]			55.3±9.3	72.2±8.6	n.s.
Number of visits of the object			12.6±7.0	45.2±24.2	n.s.
Time spent exploring the object [s]			7.4±4.70	24.0±11.1	n.s.
Latency to first visit of object [min]			4.79±2.21	1.99±0.96	n.s.
Time in corners in presence of object [min]			3.78±1.15	1.02±0.35	p <0.05

Table 1. Synopsis of behavioral parameters (arithmetic means ± SEM) of *gsk-3^{KI}* and *gsk-3^{WT}* mice.

significant in *gsk-3^{WT}* mice suggesting that *gsk-3^{KI}* mice are less sensitive to stress (Fig. 3C). The distance moved in the border zone was reduced by stress in both genotypes (Table 1). The rearings in the center were not significanty more frequent or longer in *gsk-3^{KI}* mice than in *gsk-3^{WT}* mice and tended to decrease during stress in both genotypes. The percentage of rearing mice in the center was decreased significantly more in *gsk-3^{WT}* mice (from 90.9 to 27.3 %) than in *gsk-3^{KI}* mice (from 100 to 72.7 %). The percentage of mice visiting the center, the number of visits to the center and the distance traveled within the center tended to be elevated in *gsk-3^{KI}* mice. Under stress, fewer mice visited the center and the number of visits to the center and the distance moved within the center were significantly reduced, irrespective of the genotype (Table 1).

In the light-dark box, both genotypes preferred to stay in the box (Table 1). Under stress, however, there were 4 *gsk-3^{WT}* and 1 *gsk-3^{KI}* mice that did not leave the corner in the light area they were initially placed to. For the following evaluations, these mice were excluded to avoid misinterpretation of parameters. *Gsk-3^{KI}* mice entered the dark box significantly more often than *gsk-3^{WT}* mice (Fig. 4A), a difference no more significant under stress. As in the open-field, the number and duration of rearings were significantly increased in *gsk-3^{KI}* mice as compared to *gsk-3^{WT}* mice. This was particularly true in the dark. The number of rearings in the dark was significantly decreased under stress condition, pointing to a decrease of exploratory behavior under stress (Fig. 4B). In stressed mice, the time of rearing in the light was elevated as compared to the dark box (Fig. 4C). In general, *gsk-3^{KI}* mice traveled a longer distance than *gsk-3^{WT}* mice, an effect abolished under stress conditions (Table 1).

In the O-maze, *gsk-3^{KI}* mice tended to spend more time (Fig. 5A) and travel a larger distance (Fig. 5B) in the open, unprotected area than the *gsk-3^{WT}* mice.

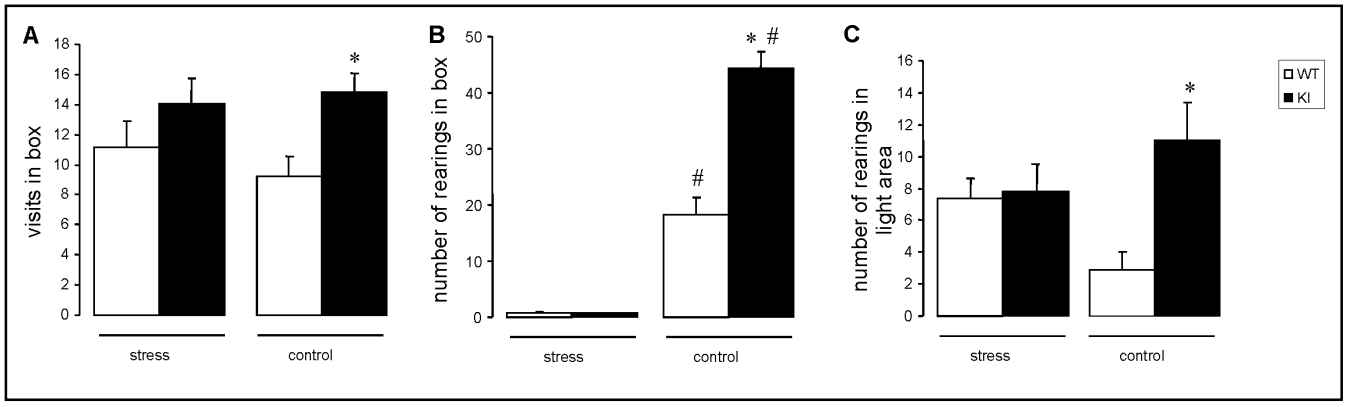

Fig. 4. Performance of *gsk-3*^{KI} and *gsk-3*^{WT} mice in the light-dark box. A. Arithmetic means ± SEM (N = 8-11 each group) of number of visits into the box. WT stress: 11.13 ± 1.77, KI stress: 14.1 ± 1.65, WT: 9.18 ± 1.37, KI: 14.82 ± 1.26. B. Arithmetic means ± SEM (N = 8-11 each group) of number of rearings in box. WT stress: 0.75 ± 0.16, KI stress: 0.7 ± 0.15, WT: 18.27 ± 3.02, KI: 44.45 ± 2.79. C. Arithmetic means ± SEM (N = 8-11 each group) of number of rearings in light area. WT stress: 7.38 ± 1.28, KI stress: 7.8 ± 1.72, WT: 2.91 ± 1.07, KI: 11 ± 2.36. * indicates a significant difference between genotypes (1-way ANOVA, $p < 0.05$). # indicates a significant difference between treatment with stress (1-way ANOVA, $p < 0.05$).

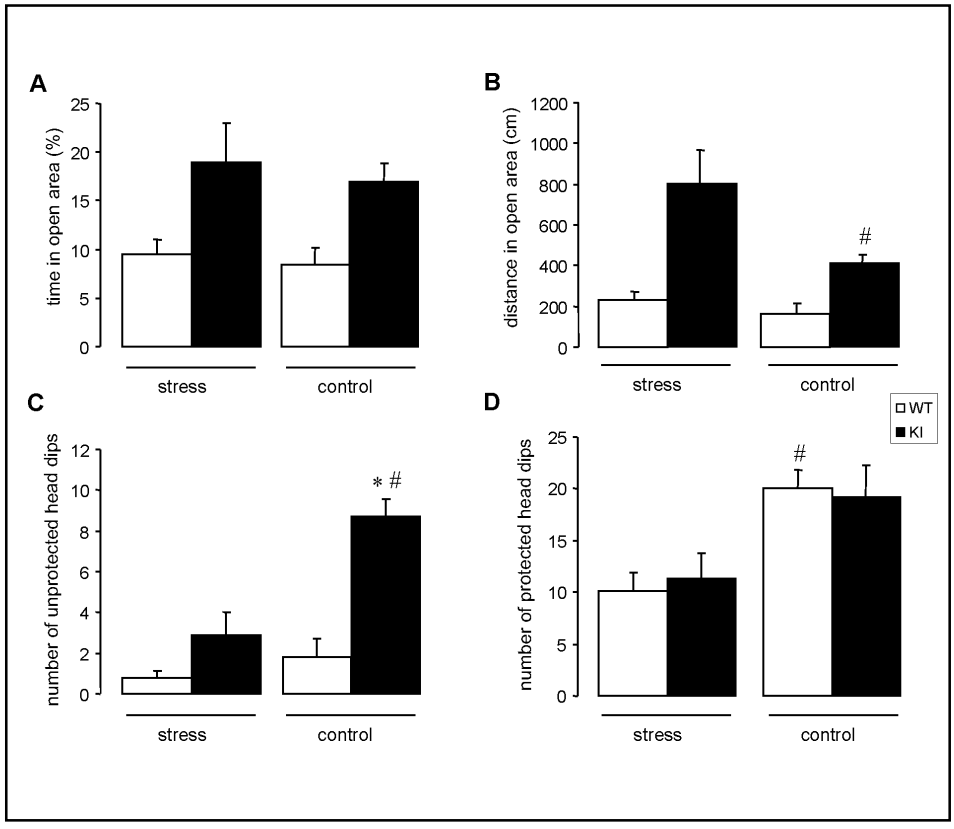

Fig. 5. Performance of *gsk-3*^{KI} and *gsk-3*^{WT} mice in the O-maze. A. Arithmetic means ± SEM (N = 11 each group) of time spent in open area. WT stress: 19.46 ± 1.57 %, KI stress: 18.97 ± 3.97 %, WT: 8.4 ± 1.81 %, KI: 16.96 ± 1.87 %. B. Arithmetic means ± SEM (N = 11 each group) of distance walked in open area. WT stress: 230.72 ± 38.8 cm, KI stress: 803.81 ± 164.65 cm, WT: 165.77 ± 52.02 cm, KI: 413.13 ± 42.48 cm. C. Arithmetic means ± SEM (N = 11 each group) of the number of unprotected head dips. WT stress: 0.82 ± 0.33, KI stress: 2.91 ± 1.12, WT: 1.82 ± 0.92, KI: 8.73 ± 0.83. D. Arithmetic means ± SEM (N = 11 each group) of the number of protected head dips. WT stress: 10.09 ± 1.83, KI stress: 11.36 ± 2.48, WT: 20.09 ± 1.78, KI: 19.27 ± 2.99. * indicates a significant difference between genotypes (1-way ANOVA, $p < 0.05$). # indicates a significant difference between treatment with stress (1-way ANOVA, $p < 0.05$).

Under stress, the tendency was the same, *gsk-3*^{KI} mice, however, walked a significantly larger distance in the open area, indicating a more pronounced hyperactivity in this environment. In the unprotected area *gsk-3*^{KI}mice looked significantly more often than *gsk-3*^{WT} mice into the deep; so-called unprotected head dips were increased in *gsk-3*^{KI} mice. The number of unprotected head dips significantly decreased in *gsk-3*^{KI} mice and tended to decrease in *gsk-3*^{WT} mice under stress conditions (Fig. 5C). The number of protected head dips, performed

while the back of the mouse was between the protecting walls, was not different between the genotypes under normal conditions, they tended to decrease in *gsk-3^{KI}* mice and they significantly decreased in *gsk-3^{WT}* mice during stress (Fig. 5D). Similar to what had been observed in the open field, the total distance traveled was significantly larger in *gsk-3^{KI}* mice than in *gsk-3^{WT}* mice (Table 1). Furthermore, the speed of stressed *gsk-3^{KI}* mice was significantly elevated (Table 1). As many as 8 out of 11 *gsk-3^{KI}* mice and only one out of 11 *gsk-3^{WT}* mice visited the opposite area. The average latency to enter the opposite area was in *gsk-3^{KI}* mice 3.26 ± 0.55 min under standard conditions and 3.72 ± 0.94 min under stress conditions and in the *gsk-3^{WT}* mouse 2.59 min under standard conditions and 8.24 min under stress conditions. In contrast, *gsk-3^{KI}* mice spent significantly less time in the area of origin than *gsk-3^{WT}* mice.

In the emergence test, a well-known home box, which was inserted into the home cage of the mouse for at least 24 h, was put into the open-field and fixed with adhesive tape. The mouse was then observed for 30 min. Both *gsk-3^{KI}* mice and *gsk-3^{WT}* mice spent more than 60% of the time in the home box area (Table 1). The distance moved in this area and the number of visits into the home box tended to be higher in *gsk-3^{KI}* mice (Table 1), pointing to enhanced activity of *gsk-3^{KI}* mice in familiar environment. Both speed and distance moved were significantly elevated in *gsk-3^{KI}* mice in the first 10 min (Fig. 6A and B). *Gsk-3^{KI}*mice then showed a significantly higher rate of habituation than *gsk-3^{WT}* mice.

In the object exploration test, the first 30 min of the experiment were identical to the emergence test. Then, a 50 ml falcon tube was put as a novel object in the arena and fixed with adhesive tape. The mouse was observed for another 30 min to see the reaction to a novel object within an otherwise familiar environment (home box). In general, mice preferred to stay in the home box, in the absence and presence of the novel object (Table 1). *Gsk-3^{KI}* mice tended to visit the novel object more often and to spend more time exploring the object which can be interpreted as increased curiosity. The latency to the first visit of the object tended to be decreased in *gsk-3^{KI}* mice indicating a faster approach to unknown objects and less fear. Furthermore, the time spent in the corner, far away from the object, was significantly increased in *gsk-3^{WT}* mice, which can be interpreted as avoidance of the object.

In the forced swimming test, *gsk-3^{KI}* mice spent significantly less time floating than *gsk-3^{WT}* mice

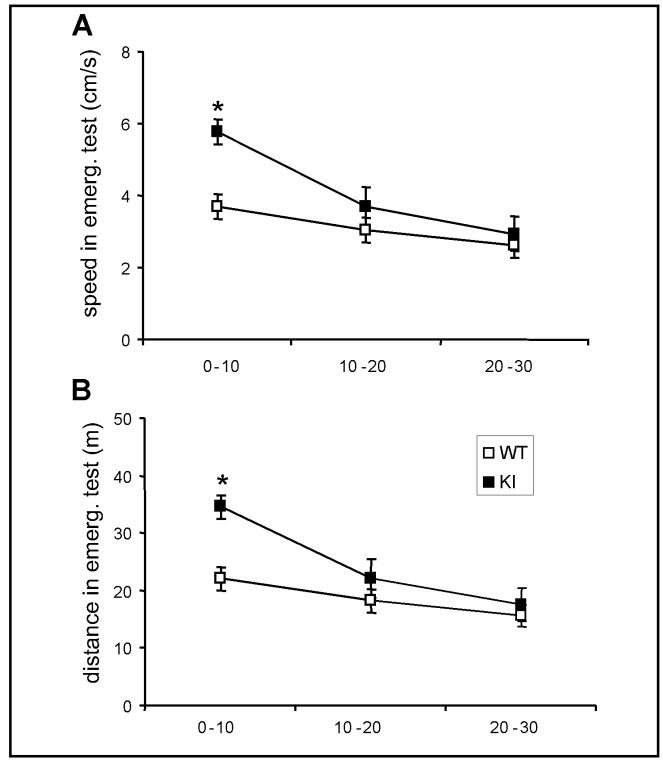

Fig. 6. Speed and distance traveled of *gsk-3^{KI}* and *gsk-3^{WT}* mice in the emergence test. A. Arithmetic means ± SEM (N = 10-11 each group) of speed in the emergence test during the first, second and third 10 minutes. KI: 5.75 ± 0.35 cm/s, 3.69 ± 0.55 cm/s, 2.94 ± 0.49 cm/s, WT: 3.68 ± 0.34 cm/s, 3.04 ± 0.35 cm/s, 2.60 ± 0.32 cm/s. B. Arithmetic means ± SEM (N = 10-11 each group) of distance traveled in the emergence test during the first, second and third 10 minutes. KI: 34.51 ± 2.07 m, 22.15 ± 3.32 m, 17.62 ± 2.93 m, WT: 22.08 ± 2.01 m, 18.22 ± 2.09 m, 15.58 ± 1.89 m. * indicates a significant difference between genotypes (1-way ANOVA, $p < 0.05$).

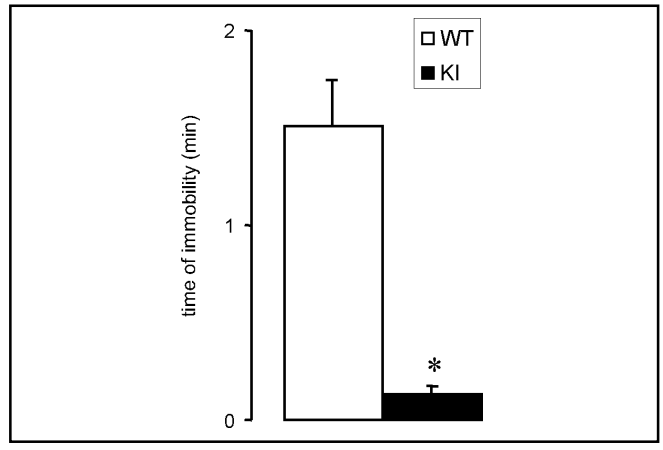

Fig. 7. Time of immobility of *gsk-3^{KI}* and *gsk-3^{WT}* mice in the forced swimming test. Arithmetic means ± SEM (N = 7 each group) of time spent floating. WT: 0.12 ± 0.05 min, KI: 1.51 ± 0.24 min. * indicates a significant difference between genotypes (1-way ANOVA, $p < 0.05$).

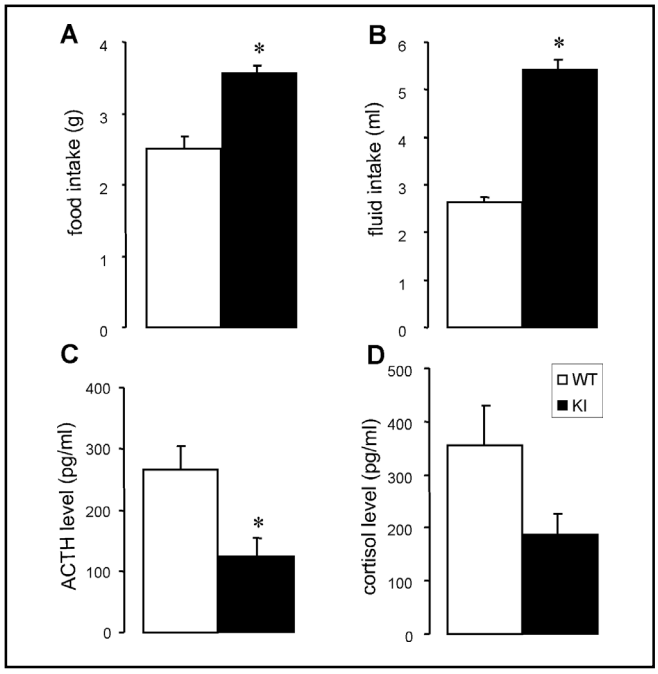

Fig. 8. Monitoring of *gsk-3^{KI}* and *gsk-3^{WT}* mice. A. Arithmetic means ± SEM (N = 11 each group) of food intake during experiments with stress. WT: 2.51 ± 0.16 g, KI: 3.56 ± 0.12 g. B. Arithmetic means ± SEM (N = 11 each group) of fluid intake during experiments with stress. WT: 2.63 ± 0.1 ml, KI: 5.42 ± 0.23 ml. C. Arithmetic means ± SEM (N = 11-13 each group) of ACTH levels. WT: 265.03 ± 39.0 pg/ml, KI: 124.5 ± 29.3 pg/ml. D. Arithmetic means ± SEM (N = 7-8 each group) of cortisol levels. WT: 356.3 ± 74.7 pg/ml, KI: 187.7 ± 39.2 pg/ml. * indicates a significant difference between genotypes (1-way ANOVA, $p < 0.05$).

(Fig. 7), which indicates a less depressive behavior.

Body weight, food and fluid intake were determined during the behavioral studies. There was no difference in body weight (Table 1), and food and fluid intake were significantly enhanced in *gsk-3^{KI}* mice (Fig. 8A and B). To determine the function of the Hypothalamic-pituitary-adrenal (HPA) axis, cortisol and adrenocorticotropic hormone (ACTH) levels were measured in *gsk-3^{WT}* and *gsk-3^{KI}* mice. ACTH was significantly decreased (Fig. 8C) and cortisol tended to be reduced (Fig. 8D) in *gsk-3^{KI}* mice.

Discussion

The present study reveals a role of PKB/SGK-dependent regulation of GSK-3 in the control of behavior. Mice carrying a mutation of GSK-3β, in which the serine of the PKB/SGK phosphorylation site was replaced by an alanine (GSK-3$\beta^{9A/9A}$) and at the same time carrying a mutation of GSK-3α, in which the serine of the PKB/SGK phosphorylation site was replaced by an alanine (GSK-3$\alpha^{21A/21A}$), are expected to be resistant to PKB/SGK-dependent regulation of GSK-3 [27]. According to the present observations, mice carrying the PKB/SGK-resistant GSK-3 mutants (*gsk-3^{KI}*) are significantly more active and curious than the corresponding wild type mice (*gsk-3^{WT}*) in several behavioral settings. Moreover, *gsk-3^{KI}* mice are less sensitive to stress and spent significantly less time floating in the forced swimming test. In line with behavioral parameters the function of the HPA axis is changed between the phenotypes: ACTH was significantly (Fig. 8C) and cortisol tended to be decreased in *gsk-3^{KI}* mice (Fig. 8D).

Several research groups formulated a hypothesis relating aberrant stress hormone regulation to causality of depression and suggested that the amount of ACTH and cortisol is significantly higher among depressive patients. Moreover, the hypothesis was raised that antidepressants act through normalization of these HPA changes, and several antidepressant treatment strategies have been developed targeting the HPA axis e.g. cortisol biosynthesis inhibitors, glucocorticoid receptor antagonists, corticotropin releasing factor receptor antagonists and vasopressin receptor antagonists [35].

According to a previous study, plasma concentrations and urinary excretions of aldosterone were similarly altered in *gsk-3^{KI}* mice [36]. Moreover, several functional parameters were different between *gsk-3^{KI}* mice and corresponding wild type mice [36]. It must be kept in mind that at least in theory, the metabolic effects of dysregulated peripheral GSK-3 may influence behavior by altering plasma metabolite, electrolyte and hormone concentrations.

The enhanced locomotor activity has been considered a correlate of mania [37, 38]. In general, mice are expected to prefer secure and safe places, represented by the border of the open-field, the box in the light-dark box experiment, the closed, tunnel-like arms in the O-maze and the home box in the emergence and object exploration test. *Gsk-3^{WT}* and *gsk-3^{KI}* mice comply with those expectations as both genotypes prefer the mentioned secure zones [39]. However, several parameters revealed increased locomotor activity in *gsk-3^{KI}* mice as measured by increased speed and total distance traveled in the open-field, light-dark box, O-maze and emergence test. The hyperactivity and increased curiosity of mice might also be evidenced by the increased number of en-

trances into the dark box [40] and the increased time spent in the entrance area of the box. However, the light-dark transition might also just reflect generally enhanced activity and the elevated dwelling time in the entrance area could also be seen as increased risk assessment of *gsk-3^{KI}* mice.

The O-maze is an established model to investigate anxiety-like behavior. Inherently, mice would avoid to pass a dangerous way as it is simulated by the open areas. The risk assessment of *gsk-3^{KI}* mice, however, did not prevent the mice from entering the opposite arm. *Gsk-3^{KI}* mice showed a clearly enhanced risk behavior while spending more time and traveling a longer distance on the open areas as well as performing more unprotected head dips. This behavior points to reduced anxiety. The fact that *gsk-3^{KI}* mice spent less time in the origin arm points to enhanced curiosity and preference to explore the environment instead of benefiting from the security in this area [41]. Moreover, reduced anxiety in *gsk-3^{KI}* mice is represented by an increased number and duration of rearings in the open-field and in the light-dark box [42].

The emergence test and object exploration test have to be seen from another point of view as the mouse is inserted into an environment which is partly familiar due to the well-known home box. During the emergence test, speed and total distance traveled were enhanced in *gsk-3^{KI}* mice, whereas there was no difference during the object exploration test. Apparently, the environment influences the behavior of mice and must be considered in the interpretation of anxiety-related behavior. The insertion of a novel object emphasized the loss of fear and the increased curiosity of *gsk-3^{KI}* mice.

As compared to *gsk-3^{WT}*, *gsk-3^{KI}* mice are clearly hyperactive in the open-field, light-dark box and O-maze. In the emergence test, hyperactivity seems less pronounced, and in the object exploration test there was no hyperactivity. This suggests that hyperactivity is novelty induced, being most pronounced, when mice are placed in a completely new environment.

The enhanced activity of the *gsk-3^{KI}* mice is reminiscent of the hyperactivity of transgenic mice overexpressing GSK-3 [24]. Unlike *gsk-3^{KI}* mice, GSK-3 transgenic mice also showed deficient habituation. Conversely, the stimulating effect of amphetamine on locomotor activity is decreased in heterozygote GSK-3 deficient mice [43] and reversed by lithium and with specific GSK-3 inhibitors [11].

Decreased sensitivity to stress, increased curiosity, decreased anxiety, decreased immobility in the forced swimming test, increased activity and decreased stress hormone levels in *gsk-3^{KI}* mice point to a depression-resistant phenotype. However, disinhibition of PI3K might lead to manic behavior, which is not common. Hypomanic states are often observed following depressive episodes and antidepressant treatment, which could result from an activation of PKB by antidepressant strategies. Indeed, antimanic treatment strategies might involve PKB-dependent regulation of GSK-3, which has been shown by several antimanic drugs (clozapine, olanzapine, lithium and valproate) [3-5]. Moreover, all of these medications show mood stabilizing properties and long-term phase prophylactic properties, which also might involve GSK-3 regulation.

Overexpression of GSK-3 in the striatum was followed by upregulation of PKB expression and downregulation of the expression of PPP2R3A, a regulatory subunit of the PKB-inactivating phosphatase PP2A [24]. Those effects were expected to inhibit GSK-3 activity and thus to mitigate the effects of GSK-3 overexpression [24]. In the *gsk-3^{KI}* mice, this negative feedback is disrupted leading to a more pronounced phenotype. The unrestrained GSK-3 activity in *gsk-3^{KI}* mice render those mice an ideal model for the in vivo testing of GSK-3 inhibitors. PKB and GSK-3 are further considered to participate in the enhanced locomotion of gene-targeted mice lacking the dopamine transporter DAT [19, 43].

As shown earlier [36], food and fluid intake were markedly enhanced in *gsk-3^{KI}* as compared to *gsk-3^{WT}* mice pointing to PKB/SGK- dependent regulation of GSK-3 in the control of food and fluid uptake. Typically, food intake is markedly changed in bipolar patients and increased food intake might be compensated by increased activity in *gsk-3^{KI}*. Moreover, disorders of food intake may parallel psychiatric diseases, such as bipolar disorder and schizophrenia [44-46]. PKB-dependent signaling in the hypothalamus is considered to mediate the food intake-lowering effect of insulin [47]. This signaling may be disrupted in the *gsk-3^{KI}* mice. PKB/SGK-dependent signaling is further required for the effect of insulin on glycogen synthase [27, 48]. Accordingly, the effect of insulin on muscle glycogen synthase is absent in *gsk-3^{KI}* mice [27]. It would be interesting to explore whether behavioral effects of hyperinsulinism are related to PKB/SGK-dependent regulation of GSK-3.

Interestingly, clozapine and olanzapine, which act on GSK-3 [49], have been shown to induce type 2 diabetes mellitus and metabolic syndrome in schizophrenic and bipolar patients [50]. Moreover, patients with schizophrenia and bipolar disorder are particularly prone to develop

obesity and type 2 diabetes mellitus [51], disorders considered to involve modulation of GSK-3 [52].

Besides insulin, brain derived neurotrophic factor (BDNF) [53, 54] activates the PI3 kinase pathway [14] and is thus expected to inhibit GSK-3β activity. BDNF has in turn been implicated in bipolar disorder [18, 55-58] and schizophrenia [59, 60].

The present study did not attempt to identify the cerebral structures involved in the altered behavior of *gsk-3^{KI}* mice. It is noteworthy, however, that in PDK1 hypomorphic mice, which display a mirror-like behavior to that of *gsk-3^{KI}* mice, GABA, taurine and serotonin concentrations were significantly decreased in the amygdala and olfactory bulb [61].

In conclusion, insensitivity of GSK-3 to the inhibitory action of PKB and SGK leads to altered food and fluid intake as well as manic-like behavior in mice. Moreover, the mutation leads to lower stress sensitivity, decreased immobility in the forced swimming test and decreased activity of the HPA axis. Thus, PKB/SGK-dependent regulation of GSK-3 activity might participate in the pathophysiology and treatment of bipolar disorders.

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