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1,25(OH)₂D₃ dependent overt hyperactivity phenotype in klotho-hypomorphic mice

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Klotho, a protein mainly expressed in kidney and cerebral choroid plexus, is a powerful regulator of 1,25(OH)₂D₃ formation. Klotho-deficient mice (*kl/kl*) suffer from excessive plasma 1,25(OH)₂D₃, Ca²⁺- and phosphate-concentrations, leading to severe soft tissue calcification and accelerated aging. NH₄Cl treatment prevents tissue calcification and premature ageing without affecting 1,25(OH)₂D₃-formation. The present study explored the impact of excessive 1,25(OH)₂D₃ formation in NH₄Cl-treated *kl/kl*-mice on behavior. To this end *kl/kl*-mice and wild-type mice were treated with NH₄Cl and either control diet or vitamin D deficient diet (LVD). As a result, plasma 1,25(OH)₂D₃, Ca²⁺- and phosphate-concentrations were significantly higher in untreated and in NH₄Cl-treated *kl/kl*-mice than in wild-type mice, a difference abrogated by LVD. In each, open field, dark-light box, and O-maze NH₄Cl-treated *kl/kl*-mice showed significantly higher exploratory behavior than untreated wild-type mice, a difference abrogated by LVD. The time of floating in the forced swimming test was significantly shorter in NH₄Cl treated *kl/kl*-mice compared to untreated wild-type mice and to *kl/kl*-mice on LVD. In wild-type animals, NH₄Cl treatment did not significantly alter 1,25(OH)₂D₃, calcium and phosphate concentrations or exploratory behavior. In conclusion, the excessive 1,25(OH)₂D₃ formation in klotho-hypomorphic mice has a profound effect on murine behavior.

Klotho is expressed mainly in the kidney, but is highly expressed as well in choroid plexus of the brain¹. The extracellular domain of the transmembrane protein may be cleaved off and enter blood or cerebrospinal fluid¹. Klotho is a powerful inhibitor of 1 α -25-hydroxyvitamin D hydroxylase (1 α hydroxylase) thus preventing 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) formation¹. Klotho influences mineral metabolism in addition by up-regulation of Ca²⁺ channels² and down-regulation of phosphate transport^{3,4}. Klotho affects further channels and transport proteins including Na⁺/K⁺-ATPase^{5,6}, Na⁺/Ca²⁺-exchanger⁷, Ca²⁺ channels⁸, K⁺ channels^{9–13} and excitatory amino acid transporters^{14,15}. Moreover, klotho counteracts inflammation^{16,17}. Klotho-hypomorphic mice (*kl/kl*) with defective promoter of the klotho gene suffer from severe tissue calcification, a wide variety of age related disorders and a severely decreased life span^{1,18}. Conversely, the life span is substantially increased in klotho overexpressing mice¹⁹. Klotho may similarly influence tissue calcification, ageing and life span of humans^{20–22}. Klotho has been implicated in the regulation of depression and cognitive function^{23–26}. Evidence has been presented pointing to an effect of klotho on oligodendrocyte maturation and myelination²⁷ and klotho has been postulated to counteract neurodegeneration²⁸. Overexpression of klotho has been shown to enhance cognition²³. Conversely, klotho deficient mice suffer from deterioration of cognitive function^{25,26,29}. The alterations of neuronal function in klotho deficient mice may, however, be due to the severe vascular calcification and may not reflect the effect of klotho or 1,25(OH)₂D₃ on cerebral function. 1,25(OH)₂D₃ has previously been shown to affect behavior^{30,31}, emotions and anxiety³². In animals, vitamin D deficiency has been shown to decrease explorative behavior and enhance anxiety, aberrant grooming, submissive social behavior, social neglect and maternal cannibalism^{33–35}. Prenatal vitamin D deficiency influences murine self-grooming behavior³⁶. Deletion of the vitamin D receptor (VDR) has similarly been shown to affect murine behavior^{34,37–42}. In humans vitamin D deficiency predisposes to several psychiatric disorders, such as depression, bipolar disorder and schizophrenia^{32,43–45}. The vitamin D receptor (VDR) and vitamin D metabolizing enzymes are expressed widely in cerebral structures including prefrontal cortex, hippocampus, cingulate gyrus, thalamus, hypothalamus, and substantia nigra⁴⁶. VDR

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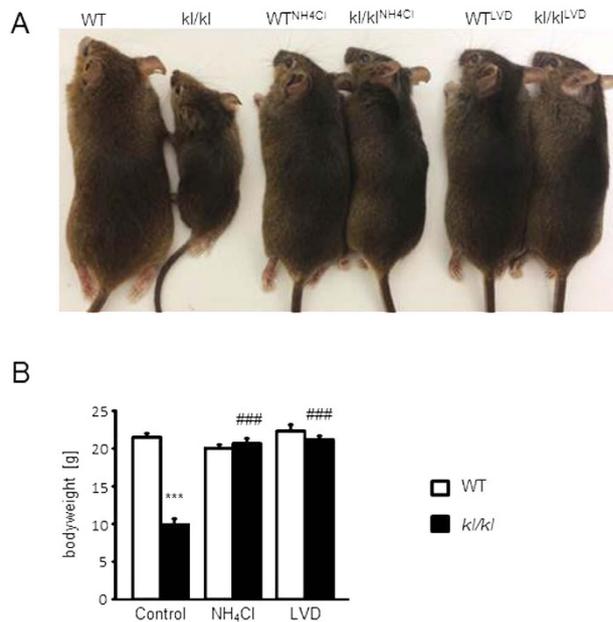


Figure 1. Effect of NH₄Cl treatment and low vitamin D diet on body weight of wild-type mice and of kl/kl mice. (A) Photograph of male wild-type mice (WT) as well as male klotho-hypomorphic mice (kl/kl) without (left) or with NH₄Cl treatment (15 g/l in drinking water) without (NH₄Cl, middle) and with (LVD, right) additional low vitamin D diet. (B) Arithmetic means \pm SEM of body weight ($n = 12\text{--}30$) of wild-type mice (WT, white bars) and kl/kl mice (kl/kl, black bars) either untreated (left bars, Control), treated with NH₄Cl solution (280 mM in drinking water) (NH₄Cl, middle bars) or treated with NH₄Cl and a vitamin D deficient diet (LVD, right bars). *** ($p < 0.001$) indicates statistically significant differences from respective wild-type mice; ### ($p < 0.001$) indicates statistically significant differences from untreated kl/kl mice. (ANOVA).

gene variants are associated with altered behavior^{47,48} as well as susceptibility to age-related changes in cognitive function and depressive symptoms⁴⁷. 1,25(OH)₂D₃ serum concentration correlates with extraversion⁴⁹, which is negatively correlated with social phobia, cluster C personality disorders and suicide risk^{45,50}. Along those lines, the seasonal variations of sun exposure and thus 1,25(OH)₂D₃ formation have been associated with seasonal affective disorders^{51–53}.

The excessive formation of 1,25(OH)₂D₃ in kl/kl mice were expected to exert profound effects on behavior. However, due to the severe vascular calcification the kl/kl mice are severely ill and not amenable to behavioral studies. Most recent observations revealed that addition of NH₄Cl into the drinking water fully prevents the severe vascular calcification and rapid ageing of kl/kl mice without affecting the excessive formation of 1,25(OH)₂D₃ and the increase of plasma phosphate and calcium concentrations⁵⁴. NH₄Cl is apparently effective by alkalinizing acidic cellular compartments which compromises the maturation of TGF β , a critical mediator of osteogenic signaling⁵⁴. Aging and life span are almost identical in NH₄Cl treated kl/kl-mice and wild type mice⁵⁴. The NH₄Cl treated kl/kl mice would thus be an ideal model to study the effect of excessive 1,25(OH)₂D₃ on behavior. Thus, kl/kl mice and wild-type mice were treated with NH₄Cl (280 mM in drinking water) and with either control diet or vitamin D deficient diet, which has previously been shown to normalize plasma 1,25(OH)₂D₃ levels in kl/kl mice⁵⁵. The behavior of those mice was explored utilizing open field, dark-light box, O-maze, and forced swimming test.

Results

Without NH₄Cl treatment, klotho-hypomorphic mice (kl/kl) suffer from a severe growth deficit (Fig. 1A). Accordingly, the body weight of kl/kl mice was significantly lower than the body weight of wild-type mice (Fig. 1B). NH₄Cl treatment increased significantly the body weight of kl/kl mice to similar values as the body weight of wild-type mice (Fig. 1B).

Plasma 1,25(OH)₂D₃ (Fig. 2A), phosphate (Fig. 2B) and Ca²⁺ (Fig. 2C) concentrations were significantly higher in untreated kl/kl mice than in wild-type mice, differences not significantly affected by NH₄Cl treatment. However, vitamin D deficient diet decreased the values of all three parameters in plasma of kl/kl mice to values similar as those in wild-type mice.

Plasma Pai-1 levels were assessed as an indicator of aging in all groups. Pai-1 levels in plasma were increased in kl/kl mice. NH₄Cl treatment and the vitamin D deficient diet normalized the plasma Pai-1 levels (Fig. 3A). As an indicator of stress, corticosterone plasma levels were determined. As a result, the plasma corticosterone levels tended to be lower in untreated and NH₄Cl treated kl/kl mice than in the respective wild type mice, differences, however, not reaching statistical significance (Fig. 3B)

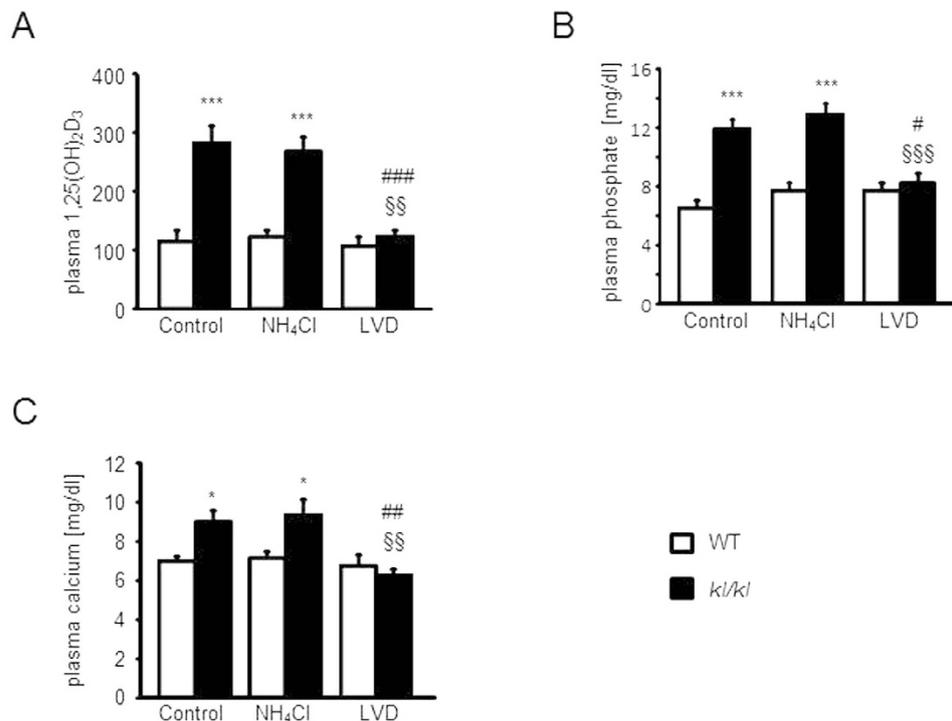


Figure 2. Effect of NH₄Cl treatment and low vitamin D diet on plasma 1,25(OH)₂D₃, phosphate, and Ca²⁺ concentrations of wild-type mice and *kl/kl* mice. (A–C) Arithmetic means \pm SEM of (A) plasma 1,25(OH)₂D₃ (n = 6), (B) phosphate (n = 12), and (C) Ca²⁺ (n = 12) concentrations of wild-type mice (WT, white bars) and *kl/kl* mice (black bars) either untreated, treated with NH₄Cl solution (280 mM in drinking water) or treated with NH₄Cl and vitamin D deficient diet (LVD, right bars). *** (p < 0.001) indicates statistically significant differences from respective wild-type mice (WT); # (p < 0.05), ## (p < 0.01), ### (p < 0.001) indicates statistically significant differences from untreated *kl/kl* mice; §§ (p < 0.01), §§§ (p < 0.001) indicates statistically significant differences from respective NH₄Cl treated mice on control diet. (ANOVA).

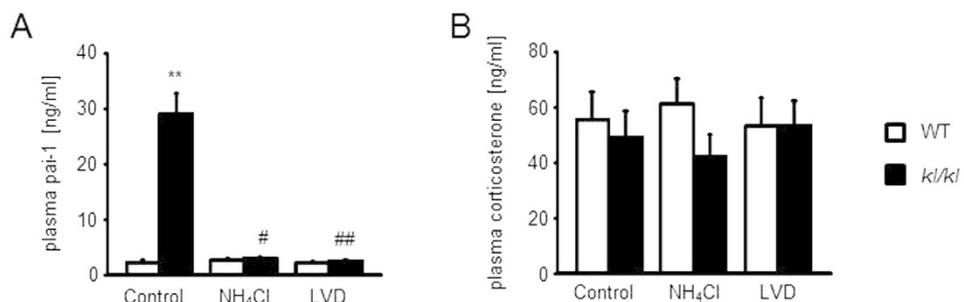


Figure 3. Effect of NH₄Cl treatment and low vitamin D diet on plasma pai-1 and corticosterone levels. (A) Arithmetic means \pm SEM (n = 8, ♂ = 4, ♀ = 4) of plasma pai-1 concentrations in wild-type mice (WT, white bars) and *kl/kl* mice (black bars) either untreated, treated with NH₄Cl solution (280 mM in drinking water) or treated with NH₄Cl and a vitamin D deficient diet (LVD, right bars). * (p < 0.05) indicates statistically significant differences from untreated wild-type mice (Control); ## (p < 0.01) indicates statistically significant differences from NH₄Cl treated *kl/kl* mice on control diet. (ANOVA). (B) Arithmetic means \pm SEM (n = 12, ♂ = 6, ♀ = 6) of plasma corticosterone concentrations of wild-type mice (WT, white bars) and *kl/kl* mice (black bars) either untreated, treated with NH₄Cl solution (280 mM in drinking water) or treated with NH₄Cl and a vitamin D deficient diet (LVD, right bars). Blood was drawn between 4 p.m. and 6 p.m.

Behavioral studies were performed with untreated control wild-type mice (Control), NH₄Cl treated WT mice and NH₄Cl treated *kl/kl* mice (NH₄Cl) under regular diet as well as WT mice and *kl/kl* mice under a vitamin D deficient diet (LVD).

In the open-field, NH₄Cl treated *kl/kl* mice seemed hyperactive which was already obvious from the recorded tracings (Fig. 4A–C). Computer analysis confirmed the visual impressions revealing significant increases in speed (Fig. 4D) and global distance travelled (Fig. 4E). The NH₄Cl treated *kl/kl* mice also spent significantly less time

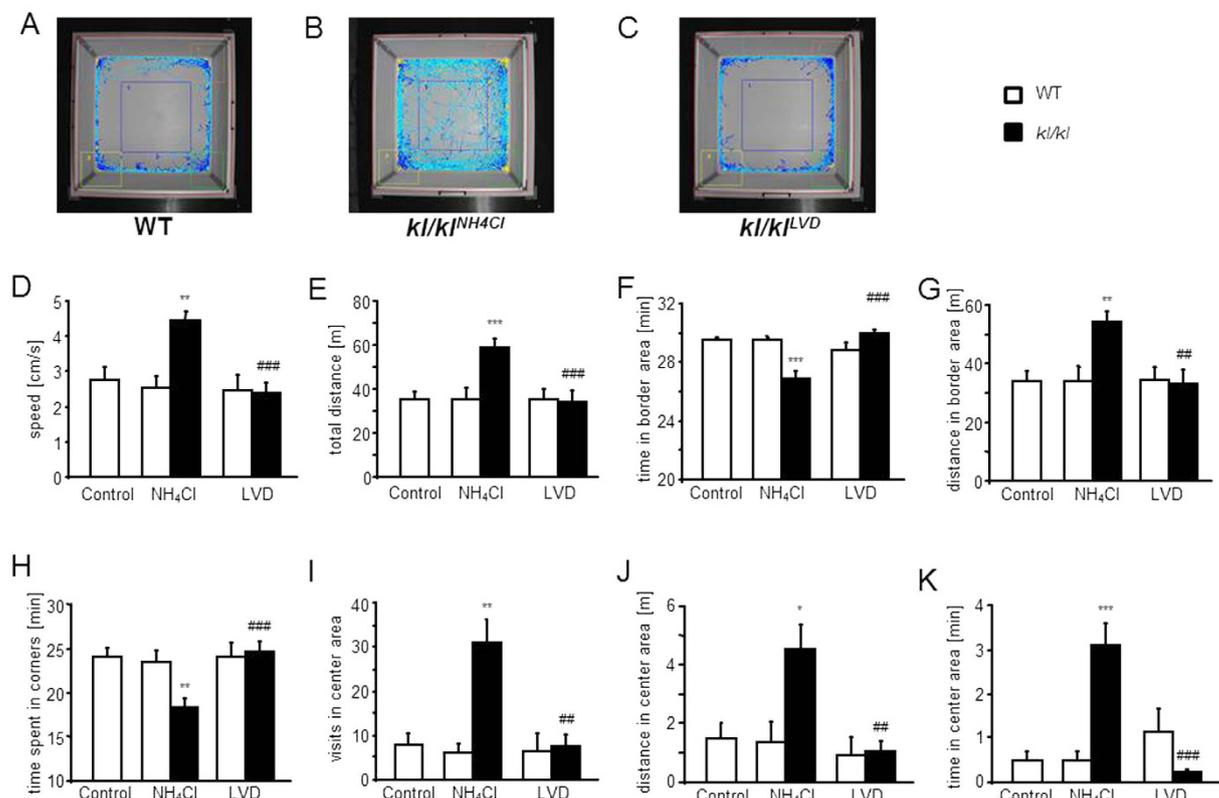


Figure 4. Effect of NH_4Cl treatment and low vitamin D diet on performance in Open Field Test.

(A–C) Photographs of the Open Field arena with representative tracings of an untreated, male wild-type mouse (WT) (A), a male, klotho-hypomorphic mouse (*kl/kl*) treated with 280 mM NH_4Cl solution (B) and a male, NH_4Cl treated *kl/kl* mouse under vitamin D deficient diet (C). (D–K) Arithmetic means \pm SEM ($n = 12\text{--}30$) of (D) average speed measured in the whole observation area, (E) total distance travelled during the observation time, (F) time spent in the border area of the Open Field arena, (G), distance travelled in the border area, (H), time spent in the corners of the Open Field arena, (I) number of visits in the center area, (J) distance travelled in the center area, (K) time spent in the center area of wild-type mice (WT, white bars) and *kl/kl* mice (*kl/kl* black bars) either untreated (Control, left bars), treated with 280 mM NH_4Cl solution (NH_4Cl , middle bars) or treated with NH_4Cl and a vitamin D deficient diet (LVD, right bars). * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) indicates statistically significant differences from untreated wild-type mice (Control); # ($p < 0.01$), ### ($p < 0.001$) indicates statistically significant differences from NH_4Cl treated *kl/kl* mice on control diet. (ANOVA).

parameter	WT	WT ^{NH₄Cl}	<i>kl/kl</i> ^{NH₄Cl}	WT ^{LVD}	<i>kl/kl</i> ^{LVD}	statistics
number of rearings in border area	84.18 \pm 14.05	68.87 \pm 15.82	138.53 \pm 10.70	60.17 \pm 13.66	78.10 \pm 11.13	P < 0.0001 ANOVA
rearing time in border area [min]	3.03 \pm 0.60	2.74 \pm 0.68	6.37 \pm 0.63	2.56 \pm 0.76	2.82 \pm 0.50	P < 0.0001 ANOVA
number of rearings in center area	1.36 \pm 0.50	1.07 \pm 0.93	7.67 \pm 1.65	1.42 \pm 0.74	0.81 \pm 0.45	P < 0.0001 nonparametric ANOVA
Rearing time in center area [s]	1.23 \pm 0.48	0.36 \pm 0.25	10.88 \pm 3.01	0.18 \pm 0.12	0.65 \pm 0.42	P < 0.0001 nonparametric ANOVA

Table 1. Synopsis of rearing parameters in the open field test (arithmetic means \pm SEM).

in the border area (Fig. 4F) but still travelled larger distances there (Fig. 4G) than wild-type mice. NH_4Cl treated *kl/kl* mice spent significantly less time in corners (Fig. 4H) and visited the center area more often (Fig. 4I) than wild-type mice. They also travelled larger distances in the center area (Fig. 4J) and spent significantly more time in that section (Fig. 4K). Interestingly, all those behavioral abnormalities were abrogated when *kl/kl* mice were fed a vitamin D deficient diet. There were no differences between untreated wild-type mice and wild-type mice treated with either NH_4Cl drinking solution or LVD. Rearing behavior is shown in Table 1.

The increased activity of NH_4Cl treated *kl/kl* mice was also apparent in the light dark transition test (Fig. 5A–C). NH_4Cl treated *kl/kl* mice spent less time in the hidden area (Fig. 5D), visited the light area more often (Fig. 5E), showed more rearings in the light area (Fig. 5F), spent more time rearing in the light area (Fig. 5G), spent more time in the entrance area of the box (Fig. 6H) and travelled larger distances in the light compartment (Fig. 5I). Although NH_4Cl treated *kl/kl* mice spent less time in the hidden area the number of rearings in the box (Fig. 5J) and the rearing time in the box (Fig. 5K) were significantly increased. Under LVD, *kl/kl* mice performed

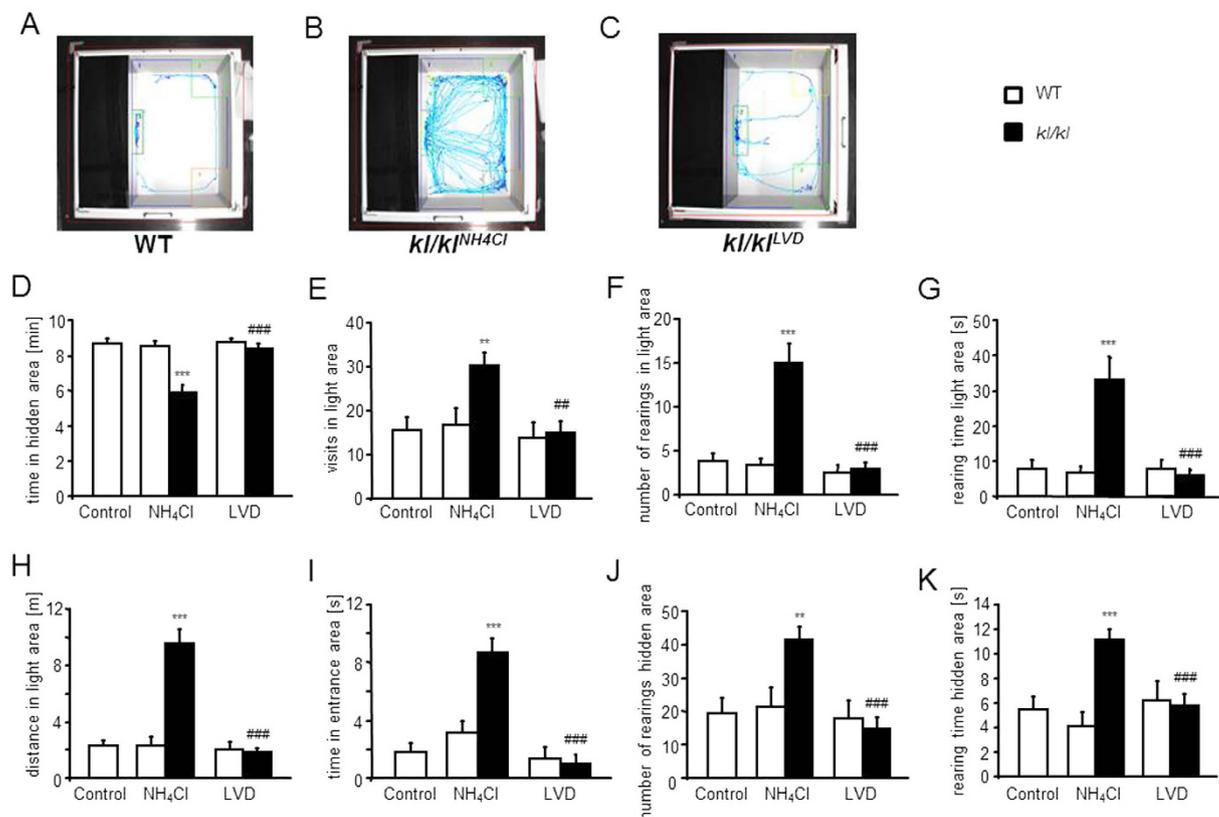


Figure 5. Effect of NH₄Cl treatment and low vitamin D diet on performance in Light Dark Box.

(A–C) Photograph of the Light Dark Box with representative tracings of an untreated male wild-type mouse (WT) (A), a male, klotho-hypomorphic mouse (*kl/kl*) treated with 280 mM NH₄Cl solution (B) and a male, NH₄Cl treated *kl/kl* mouse under vitamin D deficient diet (C). (D–K) Arithmetic means \pm SEM ($n = 12$ –30) of (D) time spent in the hidden area of the Light Dark Box arena, (E) number of visits in the light area, (F) number of rearings in the light area, (G) average rearing time in the light area, (H) distance travelled in the light area, (I) time spent in the entrance area, (J) number of rearings in the hidden area, (K) average rearing time in the hidden area of wild-type mice (WT, white bars) and *kl/kl* mice (*kl/kl* black bars) either untreated (Control, left bars), treated with 280 mM NH₄Cl solution (NH₄Cl, middle bars) or treated with NH₄Cl and a vitamin D deficient diet (LVD, right bars). **($p < 0.01$), ***($p < 0.001$) indicates statistically significant differences from untreated wild-type mice (Control); #($p < 0.01$), ###($p < 0.001$) indicates statistically significant differences from NH₄Cl treated *kl/kl* mice. (ANOVA).

like wild-type mice. Again neither NH₄Cl treatment nor LVD had an influence on the behavior of wild-type mice in the light dark transition test. Further parameters are shown in Table 2.

The recorded tracings of the O-Maze test also revealed increased activity in the NH₄Cl treated *kl/kl* mice (Fig. 6A–C). They showed significantly more protected and unprotected headspins than wild-type mice (Fig. 6D,E). NH₄Cl treated *kl/kl* mice travelled larger distances in the open areas (Fig. 6F), a differences, however, not reaching statistical significance when normalized to the total distance travelled (Fig. 6G). The ratio between distance travelled in open areas and distance travelled in closed areas tended to be higher in *kl/kl* mice, a difference, however, again not reaching statistical significance (Fig. 6H). NH₄Cl treated *kl/kl* mice spent more time in the open areas (Fig. 6I), an effect also significant when standardized to the total time spent in the open areas (Fig. 6J). Similarly the ratio of time spent in the open areas and the time spent in closed areas was significantly higher in NH₄Cl treated *kl/kl* mice (Fig. 6K) as compared to wild-type mice. Treatment with LVD abrogated the abnormal behavioral phenotype of *kl/kl* mice. In the O-Maze test neither NH₄Cl treatment nor LVD had an influence on the behavior of wild-type mice. Further parameters are shown in Table 3.

In the Forced Swimming Test the NH₄Cl treated *kl/kl* mice spent significantly less time floating on the surface of the water than wild-type mice (Fig. 7). LVD again abrogated the differences of time floating between *kl/kl* mice and wild-type mice (Fig. 7). Neither of the treatments had an effect on behavior of wild-type mice in the Forced Swimming Test.

Gender differences in the behavioral tests are apparent from Tables 4–7.

Discussion

The present observations reveal a dramatic difference between NH₄Cl treated *kl/kl* mice and NH₄Cl treated wild-type mice in several behavioral tests measuring exploratory behavior and anxiety. The difference is abrogated by vitamin D deficient diet, indicating that the excessive 1,25(OH)₂D₃ formation in *kl/kl* mice accounted for

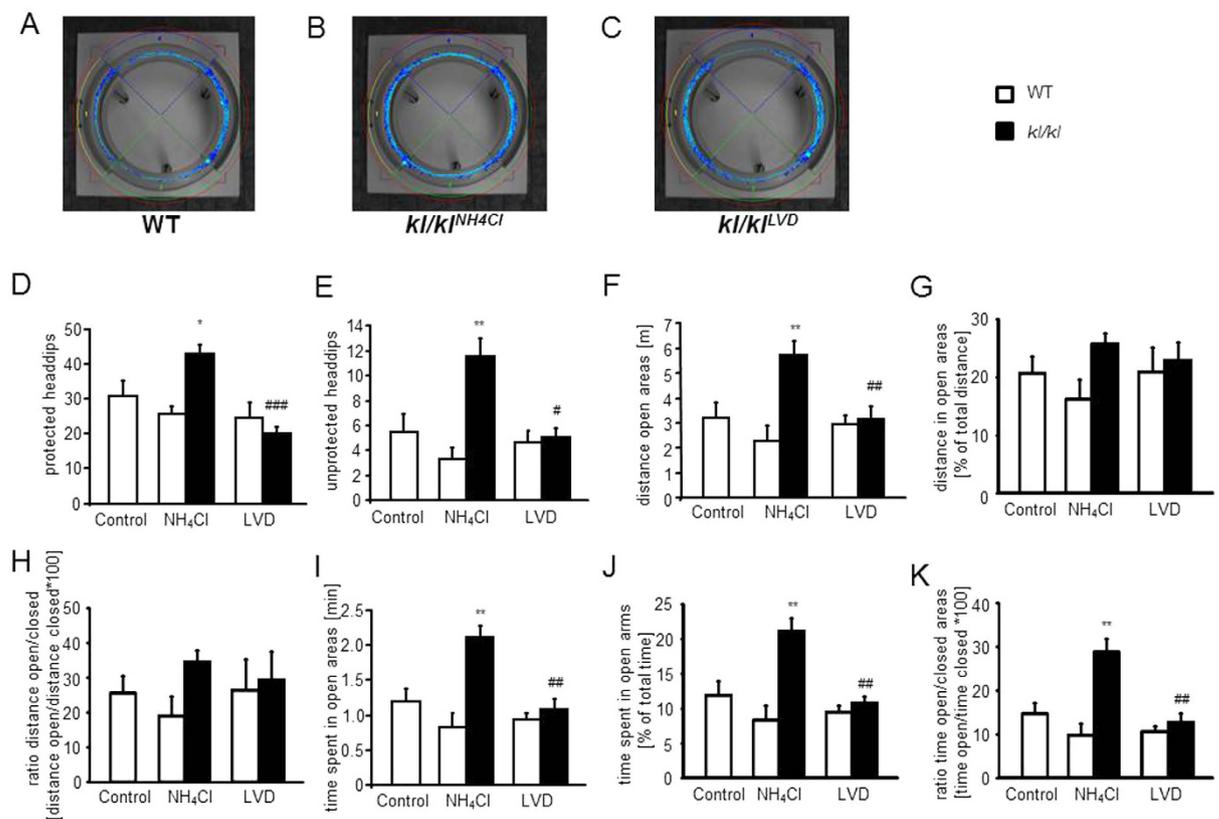


Figure 6. Effect of NH_4Cl treatment and low vitamin D diet on performance in O-Maze. (A–C) Photograph of the O-Maze with representative tracings of an untreated, male wild-type mouse (WT) (A), a male klotho-hypomorphic mouse (*kl/kl*) treated with 280 mM NH_4Cl solution (B) and a male, NH_4Cl treated *kl/kl* mouse under vitamin D deficient diet (C). (D–K) Arithmetic means \pm SEM ($n = 12\text{--}30$) of (D) number of protected headdips, (E) number of unprotected headdips, (F) distance travelled in the open areas, (G) distance travelled in the open areas as percentage of total distance, (H) ratio of distance travelled in open areas and distance travelled in closed areas, (I) time spent in open areas, (J) time spent in the open arms as percentage of total time, and (K) ratio of time spent in open arms and time spent in closed arms of wild-type mice (WT, white bars) and *kl/kl* mice (*kl/kl* black bars) either untreated (Control, left bar), treated with 280 mM NH_4Cl solution (NH_4Cl , middle bars) or treated with NH_4Cl and a vitamin D deficient diet (LVD, right bars). * ($p < 0.05$), ** ($p < 0.01$) indicates statistically significant differences from untreated wild-type mice (Control); # ($p < 0.05$), ## ($p < 0.01$), ### ($p < 0.001$) indicates statistically significant differences from NH_4Cl treated *kl/kl* mice. (ANOVA).

parameter	WT	WT ^{NH4Cl}	<i>kl/kl</i> ^{NH4Cl}	WT ^{LVD}	<i>kl/kl</i> ^{LVD}	statistics
time spent in light area [min]	1.28 \pm 0.26	1.45 \pm 0.28	4.08 \pm 0.45	1.26 \pm 0.25	1.57 \pm 0.25	$P < 0.0001$ nonparametric ANOVA
average speed [cm/s]	2.43 \pm 0.29	2.45 \pm 0.24	4.45 \pm 0.32	2.48 \pm 0.27	2.37 \pm 0.29	$P < 0.0001$ nonparametric ANOVA

Table 2. Synopsis of behavioral parameters in the Light Dark Box test (arithmetic means \pm SEM).

parameter	WT	WT ^{NH4Cl}	<i>kl/kl</i> ^{NH4Cl}	WT ^{LVD}	<i>kl/kl</i> ^{LVD}	statistics
number of visits in open areas	24.27 \pm 4.25	20.27 \pm 4.86	41.97 \pm 5.08	19.73 \pm 4.09	23.95 \pm 4.22	$P = 0.0034$ ANOVA
distance in closed areas [m]	12.41 \pm 0.82	11.81 \pm 0.77	16.58 \pm 0.77	11.01 \pm 1.35	10.65 \pm 0.79	$P < 0.0001$ ANOVA
total distance [m]	15.45 \pm 1.22	14.08 \pm 1.15	22.33 \pm 1.00	14.02 \pm 1.33	13.81 \pm 0.96	$P < 0.0001$ ANOVA
average speed [cm/s]	2.74 \pm 0.30	2.37 \pm 0.22	3.63 \pm 0.18	1.99 \pm 0.14	2.40 \pm 0.17	$P < 0.0001$ nonparametric ANOVA

Table 3. Synopsis of behavioral parameters in the O Maze test (arithmetic means \pm SEM).

the observed differences between NH_4Cl treated *kl/kl* mice and wild-type mice. The observations do not rule out more direct effects of klotho deficiency but indicate that the observed differences are in large part explained by

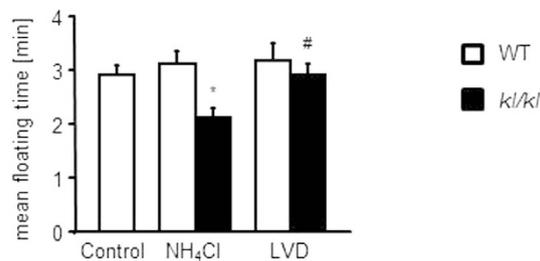


Figure 7. Effect of NH₄Cl treatment and low vitamin D diet on performance in Forced Swimming Test. Arithmetic means \pm SEM (n = 12–30) of floating time of wild-type mice (WT, white bars) and *kl/kl* mice (*kl/kl* black bars) either untreated (Control, left bar), treated with 280 mM NH₄Cl solution (NH₄Cl, middle bars) or treated with NH₄Cl and a vitamin D deficient diet (LVD, right bars). * (p < 0.05) indicates statistically significant differences from untreated wild-type mice (Control); # (p < 0.05) indicates statistically significant differences from NH₄Cl treated *kl/kl* mice. (ANOVA).

parameter		WT	WT ^{NH₄Cl}	<i>kl/kl</i> ^{NH₄Cl}	WT ^{LVD}	<i>kl/kl</i> ^{LVD}
speed [cm/s]	♂	3.00 \pm 0.58	2.75 \pm 0.39	4.86 \pm 0.47	2.37 \pm 0.68	2.51 \pm 0.40
	♀	2.55 \pm 0.41	2.47 \pm 0.58	4.11 \pm 0.29	2.53 \pm 0.64	2.24 \pm 0.45
	ttest	0.5266	0.5381	0.1651	0.8619	0.6612
total distance [m]	♂	37.71 \pm 6.95	36.03 \pm 5.58	61.13 \pm 6.53	35.91 \pm 7.44	33.81 \pm 6.45
	♀	33.09 \pm 3.30	32.27 \pm 9.82	56.84 \pm 5.37	34.74 \pm 6.83	35.34 \pm 7.33
	ttest	0.5553	0.9000	0.6128	0.9103	0.8762
time in border area [min]	♂	29.66 \pm 0.10	29.48 \pm 0.40	27.47 \pm 0.75	29.28 \pm 0.32	29.86 \pm 0.05
	♀	29.35 \pm 0.39	29.62 \pm 0.20	26.43 \pm 0.66	28.45 \pm 0.99	29.71 \pm 0.15
	ttest	0.4611	0.8112	0.3062	0.4423	0.3597
distance in border area [m]	♂	36.31 \pm 6.39	34.29 \pm 5.93	58.04 \pm 5.78	34.40 \pm 4.56	33.03 \pm 6.28
	♀	34.50 \pm 3.45	31.39 \pm 9.61	51.21 \pm 4.70	34.40 \pm 6.77	34.04 \pm 6.77
	ttest	0.5153	0.9585	0.3627	1	0.9141
time spent in corners [min]	♂	24.03 \pm 1.62	22.33 \pm 2.05	18.96 \pm 0.88	23.24 \pm 2.32	25.41 \pm 1.12
	♀	23.97 \pm 1.58	24.16 \pm 1.61	17.94 \pm 1.62	24.91 \pm 2.39	23.95 \pm 2.13
	ttest	0.9805	0.3798	0.6167	0.6276	0.5417
distance in center area [m]	♂	1.48 \pm 0.69	1.74 \pm 1.28	5.63 \pm 1.32	1.35 \pm 1.26	0.77 \pm 0.39
	♀	1.60 \pm 0.86	0.87 \pm 0.48	3.09 \pm 0.85	0.49 \pm 0.17	1.30 \pm 0.68
	ttest	0.8627	0.5802	0.1446	0.5154	0.4969
visits in center area	♂	5.73 \pm 1.75	5.13 \pm 2.75	23.39 \pm 6.39	9.67 \pm 7.97	5.82 \pm 2.68
	♀	10.18 \pm 4.68	7.25 \pm 3.44	36.53 \pm 8.42	3.00 \pm 1.75	9.60 \pm 4.74
	ttest	0.3837	0.6284	0.2482	0.4329	0.4854
time in center area [min]	♂	0.35 \pm 0.10	0.53 \pm 0.40	2.53 \pm 0.75	0.72 \pm 0.32	0.14 \pm 0.05
	♀	0.65 \pm 0.39	0.39 \pm 0.20	3.57 \pm 0.66	1.56 \pm 0.99	0.29 \pm 0.15
	ttest	0.4611	0.8112	0.3062	0.4423	0.3597
number of rearings in border area	♂	96.73 \pm 19.95	68.50 \pm 15.56	154.69 \pm 15.97	68.50 \pm 24.14	86.82 \pm 15.65
	♀	71.64 \pm 20.00	69.75 \pm 30.48	126.18 \pm 14.07	51.83 \pm 14.50	68.50 \pm 16.09
	ttest	0.3850	0.9813	0.1916	0.5671	0.4251
rearing time in border area [min]	♂	3.22 \pm 0.81	3.18 \pm 1.00	7.56 \pm 0.91	2.59 \pm 1.14	3.10 \pm 0.67
	♀	2.83 \pm 0.91	2.33 \pm 0.96	6.37 \pm 0.63	2.56 \pm 1.13	2.52 \pm 0.79
	ttest	0.7506	0.5128	0.1016	0.9865	0.5800
number of rearings in center area	♂	1.46 \pm 0.78	1.75 \pm 1.05	5.69 \pm 1.67	1.67 \pm 1.31	0.27 \pm 0.20
	♀	1.27 \pm 0.68	0.25 \pm 0.29	9.18 \pm 2.59	1.17 \pm 0.83	1.4 \pm 0.95
	ttest	0.8618	0.4543	0.3019	0.7538	0.2364
rearing time in center area [min]	♂	1.36 \pm 0.63	0.28 \pm 0.20	6.92 \pm 2.05	0.18 \pm 0.16	0.34 \pm 0.23
	♀	1.10 \pm 0.76	0.40 \pm 0.36	13.91 \pm 5.03	0.18 \pm 0.17	0.99 \pm 0.86
	ttest	0.7945	0.7266	0.2573	1	0.4501

Table 4. Differences between male and female mice in the open field test (arithmetic means \pm SEM).

parameter		WT	WT ^{NH4Cl}	kl/kl ^{NH4Cl}	WT ^{LVD}	kl/kl ^{LVD}
time in hidden area [min]	♂	8.67 ± 0.48	8.71 ± 0.29	6.26 ± 0.57	8.56 ± 0.38	8.47 ± 0.40
	♀	8.79 ± 0.23	8.37 ± 0.53	5.66 ± 0.67	8.91 ± 0.35	8.39 ± 0.32
	ttest	0.8226	0.5645	0.5234	0.5208	0.8785
visits in light area	♂	13.91 ± 3.69	15.75 ± 5.23	26.92 ± 3.69	11.67 ± 3.48	14.36 ± 3.92
	♀	17.10 ± 5.23	17.57 ± 5.75	32.88 ± 4.04	15.67 ± 6.56	15.40 ± 4.16
	ttest	0.6217	0.8180	0.3000	0.6017	0.8579
number of rearings in light area	♂	3.55 ± 1.06	3.63 ± 1.43	16.12 ± 3.32	3.50 ± 1.63	3.00 ± 0.62
	♀	4.27 ± 1.18	3.00 ± 1.07	13.54 ± 2.85	1.67 ± 0.72	3.10 ± 1.34
	ttest	0.6516	0.7377	0.5747	0.3268	0.9449
rearing time light area [s]	♂	8.69 ± 4.40	5.37 ± 2.14	33.31 ± 6.41	5.66 ± 2.86	4.34 ± 1.31
	♀	7.20 ± 2.33	8.51 ± 2.45	33.32 ± 11.67	6.06 ± 2.41	8.13 ± 2.86
	ttest	0.7673	0.3493	0.9985	0.9166	0.2280
distance in light area [m]	♂	2.51 ± 0.55	2.27 ± 0.77	8.870 ± 1.05	1.77 ± 0.57	1.90 ± 0.45
	♀	2.17 ± 0.39	2.33 ± 1.09	10.27 ± 1.58	2.33 ± 0.96	1.84 ± 0.40
	ttest	0.6217	0.9659	0.4444	0.6257	0.9242
time in entrance area [min]	♂	1.55 ± 0.93	3.46 ± 1.17	9.24 ± 1.31	1.39 ± 0.83	1.05 ± 0.75
	♀	1.99 ± 0.03	2.71 ± 1.32	8.25 ± 1.44	1.97 ± 1.66	0.57 ± 0.35
	ttest	0.7532	0.6746	0.6248	0.5077	0.6521
number of rearings in hidden area	♂	21.33 ± 6.29	20.38 ± 7.01	41.35 ± 5.39	16.67 ± 5.10	15.46 ± 5.42
	♀	19.82 ± 6.27	22.86 ± 9.45	41.52 ± 6.01	18.67 ± 10.27	14.00 ± 4.70
	ttest	0.8665	0.8335	0.9444	0.8650	0.8430
rearing time hidden area [s]	♂	5.51 ± 1.07	3.91 ± 1.50	11.19 ± 0.85	6.34 ± 1.82	5.24 ± 1.32
	♀	5.79 ± 1.42	4.41 ± 1.86	11.40 ± 1.07	6.11 ± 2.70	6.34 ± 1.59
	ttest	0.7965	0.8361	0.8345	0.9474	0.5904
time in light area [min]	♂	1.34 ± 0.48	1.29 ± 0.29	3.74 ± 0.57	1.44 ± 0.38	1.53 ± 0.40
	♀	1.22 ± 0.23	1.64 ± 0.53	4.34 ± 0.67	1.09 ± 0.35	1.62 ± 0.32
	ttest	0.8226	0.5645	0.5234	0.5208	0.8785
speed [cm/s]	♂	3.45 ± 0.43	2.41 ± 0.36	4.53 ± 0.38	2.95 ± 0.36	2.23 ± 0.36
	♀	2.40 ± 0.41	2.49 ± 0.33	4.39 ± 0.50	2.03 ± 0.34	2.52 ± 0.48
	ttest	0.9335	0.8677	0.8353	0.0948	0.6244

Table 5. Differences between male and female mice in the Light Dark Box test (arithmetic means ± SEM).

excessive formation of 1,25(OH)₂D₃. The effects are, however, not necessarily due to a direct effect of 1,25(OH)₂D₃ on neuronal function and behavior.

NH₄Cl treatment had no significant effect in wildtype mice indicating that the NH₄Cl treatment does not alter any of the measured parameters on its own. Similar to earlier observations⁵⁴, NH₄Cl treatment did not appreciably influence plasma 1,25(OH)₂D₃, Ca²⁺ and phosphate concentrations. NH₄Cl interferes with osteogenic signaling thus preventing the disastrous tissue calcification in *kl/kl* mice⁵⁴.

The present observations underscore the powerful direct or indirect influence of 1,25(OH)₂D₃ on the brain, which presumably accounts for the various cerebral effects of vitamin D deficiency. Decreased serum levels of the 1,25(OH)₂D₃ precursor 25(OH)D₃ were found in patients suffering from depression^{56,57}. Conversely, vitamin D supplementation has been reported to counteract depressive symptoms^{51–53}. Vitamin D deficiency during brain development is apparently a risk factor for the development of schizophrenia, a condition associated with enhanced neuroticism and decreased extraversion⁵⁸. Conversely vitamin D supplementation decreases the risk to develop psychotic-like symptoms⁴⁴.

The present observations did not address the mechanisms underlying the altered behavior of *kl/kl* mice. Several mechanisms have been suggested to participate in the cerebral effects of 1,25(OH)₂D₃, including antioxidant effects, inhibition of inflammation and vascular injury, stimulation of neurotrophins and improvement of metabolic and cardiovascular function³⁰. Vitamin D deficiency has been suggested to modify cellular development, dopamine metabolism, and brain morphology⁵⁹. In theory, 1,25(OH)₂D₃ could affect neuronal function by influencing neuronal or glial cytosolic Ca²⁺ activity^{60–62}. 1,25(OH)₂D₃ may interfere with the cerebral action of glucocorticoids, which are involved in the development of major depression⁶³. 1,25(OH)₂D₃ dependent calcium binding protein has been observed in nuclei influencing the pineal gland⁶⁴ and vitamin D₃ deficiency may contribute to the desynchronisation in seasonal affective disorders⁶⁵.

In wild type animals, dietary vitamin D does not necessarily influence 1,25(OH)₂D₃ concentration, as 1 α -25-hydroxyvitamin D hydroxylase and thus 1,25(OH)₂D₃ formation is under tight regulation by FGF23 and *klotho*¹. Both, FGF23 and *klotho* expression are stimulated by 1,25(OH)₂D₃ and thus 1,25(OH)₂D₃ formation is limited by negative feedback regulation^{1,66,67}. In the presence of *klotho* and FGF23, the diet becomes critically important only during vitamin D deficiency. The negative feedback is missing in *kl/kl* mice and in those mice the formation of 1,25(OH)₂D₃ is a function of dietary vitamin D even at excessive 1,25(OH)₂D₃

parameter		WT	WT ^{NH4Cl}	kl/kl ^{NH4Cl}	WT ^{LVD}	kl/kl ^{LVD}
protected headdips	♂	27.00 ± 6.15	23.88 ± 2.86	45.65 ± 3.65	22.83 ± 5.06	20.50 ± 2.66
	♀	34.27 ± 6.42	29.75 ± 3.76	41.00 ± 3.77	26.50 ± 7.21	19.82 ± 2.93
	ttest	0.4231	0.4412	0.4735	0.6858	0.8660
unprotected headdips	♂	4.36 ± 1.50	3.50 ± 1.35	11.77 ± 1.71	5.67 ± 1.45	5.40 ± 1.32
	♀	6.64 ± 2.44	2.75 ± 1.30	11.47 ± 2.14	3.67 ± 1.17	4.73 ± 0.95
	ttest	0.4365	0.8528	0.9350	0.3095	0.6799
visits in open arms	♂	27.27 ± 7.36	18.63 ± 7.10	44.14 ± 5.10	19.50 ± 3.23	22.00 ± 4.69
	♀	21.27 ± 4.46	19.88 ± 7.06	39.59 ± 6.95	20.00 ± 8.74	25.73 ± 7.02
	ttest	0.4937	0.7324	0.5636	0.9552	0.6705
distance in open arms [m]	♂	3.46 ± 1.02	2.24 ± 0.97	6.03 ± 0.75	3.28 ± 0.41	2.98 ± 0.82
	♀	2.96 ± 0.73	2.03 ± 0.82	5.53 ± 0.76	2.57 ± 0.62	3.32 ± 0.66
	ttest	0.6895	0.9564	0.6933	0.3616	0.7462
time in open arms [min]	♂	1.11 ± 0.30	0.73 ± 0.27	2.02 ± 0.26	0.94 ± 0.12	1.12 ± 0.21
	♀	1.27 ± 0.25	0.82 ± 0.33	2.18 ± 0.25	0.96 ± 0.15	1.03 ± 0.24
	ttest	0.6783	0.6649	0.6700	0.9447	0.7722
distance in closed arms [m]	♂	12.42 ± 1.00	12.07 ± 1.03	15.65 ± 0.76	11.69 ± 2.32	10.45 ± 1.26
	♀	12.40 ± 1.34	11.26 ± 1.25	17.92 ± 1.23	10.49 ± 1.56	10.84 ± 1.03
	ttest	0.9898	0.7379	0.3923	0.6770	0.8103
total distance [m]	♂	15.56 ± 1.88	14.30 ± 1.62	21.68 ± 1.22	14.92 ± 2.31	13.42 ± 1.64
	♀	15.35 ± 1.63	13.30 ± 1.76	22.82 ± 1.51	13.12 ± 1.46	14.16 ± 1.13
	ttest	0.9336	0.8454	0.6513	0.5265	0.7114
speed [cm/s]	♂	2.29 ± 0.30	2.22 ± 0.29	3.67 ± 0.20	2.01 ± 0.29	2.47 ± 0.25
	♀	3.82 ± 0.49	2.43 ± 0.34	3.63 ± 0.29	1.97 ± 0.09	2.33 ± 0.23
	ttest	0.1353	0.4781	0.8705	0.8882	0.6823
time in open arms [%]	♂	11.06 ± 2.99	7.34 ± 2.74	20.17 ± 2.55	9.41 ± 1.23	11.22 ± 2.10
	♀	12.70 ± 2.51	8.17 ± 3.33	21.76 ± 2.47	9.55 ± 1.46	10.28 ± 2.39
	ttest	0.6783	0.6649	0.6696	0.9447	0.7721
time open/closed arms *100	♂	13.75 ± 3.94	8.67 ± 3.57	26.89 ± 4.27	10.50 ± 1.54	13.21 ± 2.68
	♀	15.56 ± 3.54	9.85 ± 4.48	30.25 ± 4.88	10.70 ± 1.78	12.35 ± 3.33
	ttest	0.7361	0.6674	0.6428	0.9328	0.8439
distance open arms [%]	♂	22.24 ± 4.33	15.62 ± 5.12	27.81 ± 2.83	22.01 ± 2.73	22.18 ± 4.81
	♀	19.26 ± 3.96	15.29 ± 4.79	24.21 ± 2.24	19.60 ± 8.08	23.45 ± 4.34
	ttest	0.6184	0.7854	0.5286	0.4239	0.6645
distance open/closed arms *100	♂	27.87 ± 6.49	18.52 ± 9.19	38.53 ± 5.27	28.09 ± 4.35	28.50 ± 9.67
	♀	23.86 ± 6.95	18.05 ± 7.11	31.95 ± 4.30	24.52 ± 7.26	30.64 ± 12.81
	ttest	0.6544	0.9178	0.4911	0.2703	0.6927

Table 6. Differences between male and female mice in the O Maze test (arithmetic means ± SEM).

concentrations. In view of the present observation any regulator of FGF23 and/or *klotho* expression or any regulator of 1α -25-hydroxyvitamin D hydroxylase may be expected to impact on exploratory behavior. In this respect it is noteworthy that *klotho* is downregulated and $1,25(\text{OH})_2\text{D}_3$ formation up-regulated by dehydration⁶⁸ and parathyroid hormone⁶⁹, FGF23 is up-regulated and $1,25(\text{OH})_2\text{D}_3$ formation downregulated by lithium^{70,71} and 1α -25-hydroxyvitamin D hydroxylase inhibited by CO-releasing molecule CORM-2⁷².

In conclusion, the present observations reveal that disruption of *klotho* dependent inhibition of 1α -25-hydroxyvitamin D hydroxylase and thus excessive $1,25(\text{OH})_2\text{D}_3$ formation leads to profound stimulation of exploratory behavior.

Materials and Methods

Mice. All animal experiments were conducted according to the German law for the welfare of animals and were approved by local authorities (Regierungspräsidium Tübingen). The methods were carried out in accordance with the approved guidelines. The original *klotho*-hypomorphic (*kl/kl*) mice were generated by Kuro-o *et al.*¹⁹. In an attempt to insert the rabbit type-I Na^+/H^+ exchanger via a standard microinjection method into the genome of the mice, the promoter region of the *klotho* gene was disrupted. The mice do not express the expected transgene but cross-breeding of the heterozygous mice resulted in animals homozygous for the insertional mutation and a severe aging-like phenotype. RT-PCR analysis revealed that *klotho* is still expressed to a low extent and therefore the mice are referred to as *klotho*-hypomorphic mice. The original *kl/kl* mice had a mixed background of C57BL/6J and C3H/J. Congenic strains of *kl/kl* mice were produced by repeated backcrosses (>9 generations) to the 129Sv inbred strain and used in this study. The mice were generated from heterozygous breedings, and male and female *kl/kl* mice were compared to male and female wild-type (WT) mice⁵⁴. The animals were housed

parameter		WT	WT ^{NH4Cl}	kl/kl ^{NH4Cl}	WT ^{LVD}	kl/kl ^{LVD}
mean floating time [min]	♂	3.10 ± 0.23	3.01 ± 0.33	2.20 ± 0.36	2.93 ± 0.61	2.81 ± 0.30
	♀	2.70 ± 0.30	3.15 ± 0.35	2.02 ± 0.24	3.40 ± 0.29	3.01 ± 0.26
	ttest	0.3018	0.9084	0.6702	0.5035	0.7693

Table 7. Differences between male and female mice in the Forced Swimming test (arithmetic means ± SEM).

	total number of animals	number of ♂	number of ♀
kl/kl ^{NH4Cl}	30	13	17
kl/kl ^{LVD}	21	10	11
WT ^{NH4Cl}	15	8	7
WT	22	11	11
WT ^{LVD}	12	6	6

Table 8. Number of animals used in the experiment.

in groups of 2–6 mice per cage. The temperature was set to $22 \pm 2^\circ\text{C}$ and the humidity was $55 \pm 10\%$. The mice had access to either tap water or a solution of NH_4Cl in tap water (280 mM) ad libitum and were fed either a standard chow diet (Altromin C1000) or a vitamin D deficient diet (Altromin C1017). The lifelong NH_4Cl treatment started with the mating of the parental generation and was maintained from pregnancy until the end of the experiment. The animals were maintained at a 12:12 h inverted cycle with lights on between 7 p.m. and 7 a.m. 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 rack was brought to the test room at least 30 min before each experiment and dry surfaces of apparatus were thoroughly cleaned with 70% ethanol before releasing the animal. Experiments extended over a total of 4 months, the age was 10–11 weeks at the beginning and 6 months at the end of the experiments. Untreated *kl/kl* mice could not be used in the behavioral tests because of their poor physical condition (Table 8).

Blood chemistry. Blood specimens were obtained the day after the completion of the behavioral studies between 4–6 p.m. by puncturing the retro-orbital plexus. Plasma phosphate and calcium concentrations were determined utilizing a photometric method (FUJI FDC 3500i, Sysmex, Norsted, Germany). The plasma $1,25(\text{OH})_2\text{-vitamin D}_3$ (IDS, Boldon, UK), corticosterone (DRG, Marburg, Germany) and Pai 1 (Molecular Innovations, Novi, USA) concentrations were measured by ELISA.

Behavioral studies. For data acquisition, animals were video tracked by the camera 302050-SW-KIT-2-CAM at a resolution of 0.62 to 0.72 pixel (TSE-Systems, Bad Homburg, Germany). Raw data were transferred to Microsoft Excel for further analysis.

Tests were done in the following order: Open-field, light-dark box, O-maze, and forced swimming test. Experiments were performed with diffuse indirect room light produced by dimmable bulbs, adjusted to yield approximately 12 lux in the center of the experimental arena. The only exception was the light-dark-box test where full room light was switched on to obtain approximately 500 lux in the lit chamber. The experiments have been performed as described previously in detail⁷³.

For open-field the quadratic open-field arena had a side length of 50 cm, a white plastic floor, and 40 cm high sidewalls made of white polypropylene. Rearing behavior was assessed by a metallic frame surrounding the arena generating a photoelectric barrier (vertical activity). A border area was considered with a width of 10 cm from the wall dividing the arena in a center and a border area. Each subject was released near the wall and observed for 30 min.

For the light-dark box a 40 cm black acryl box was inserted in the open-field arena, which covered 33% of the surface area. An aperture of 10 cm length and 11 cm height with rounded down corners led into the dark box. Each subject was released in the same corner of the illuminated compartment and observed for 10 min⁷⁴.

For O-maze a 5.5 cm wide annular runway was constructed using grey plastic. It had an outer diameter of 46 cm and was placed inside the above open-field arena 40 cm above the floor^{73,75}. The two opposing 90° closed sectors were protected by 11 cm high inner and outer walls of grey polyvinyl-chloride, while the remaining two open sectors had no walls. Animals were released in one of the closed sectors and observed for 10 min. Over time, the animal's exploratory drive competes with their natural avoidance of heights. The mice start to explore the cliff by dipping their heads. As an additional parameter the number of headdips was counted. Differentiated were protected headdips, when the headdips occurred with the mice still in the protected zone, and the unprotected headdips, when the mice left the protected zone completely to explore the cliff. The numbers of headdips were counted manually.

In the forced swimming test mice were placed in a container filled with water of temperatures between 24 and 26°C. The diameter of the container was 20 cm. The mice were 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 recorded⁷⁶.

Statistics. Data are provided as means \pm SEM, n represents the number of independent experiments. All data were tested for significance using parametric ANOVA followed by Tukey-Kramer Multiple Comparisons Test in case of equal standard deviations (tested with Bartlett's) or nonparametric ANOVA (Kruskal-Wallis Test) in case of significant differences in standard deviations followed by Dunn's Multiple Comparison Test. Only results with $p < 0.05$ were considered statistically significant. The statistical calculations were performed utilizing the Graph Pad Prism software.

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Author Contributions

C.B.L., J.V., M.K. and U.E.L. performed experiments and analyzed data. F.L. and U.E.L. wrote the paper. All authors reviewed the manuscript and approved of submission.

Additional Information

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