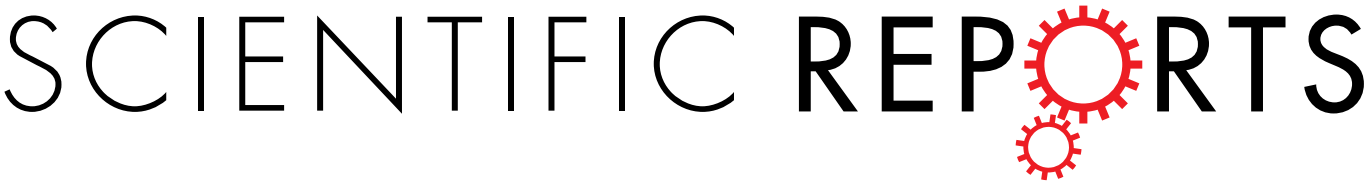

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# $1,25(OH)_2D_3$ dependent overt hyperactivity phenotype in klotho-hypomorphic mice

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**Klotho, a protein mainly expressed in kidney and cerebral choroid plexus, is a powerful regulator of $1,25(OH)_2D_3$ formation. Klotho-deficient mice (*kl/kl*) suffer from excessive plasma $1,25(OH)_2D_3$-, $Ca^{2+}$- and phosphate-concentrations, leading to severe soft tissue calcification and accelerated aging. $NH_4Cl$ treatment prevents tissue calcification and premature ageing without affecting $1,25(OH)_2D_3$-formation. The present study explored the impact of excessive $1,25(OH)_2D_3$ formation in $NH_4Cl$-treated *kl/kl*-mice on behavior. To this end *kl/kl*-mice and wild-type mice were treated with $NH_4Cl$ and either control diet or vitamin D deficient diet (LVD). As a result, plasma $1,25(OH)_2D_3$-, $Ca^{2+}$- and phosphate-concentrations were significantly higher in untreated and in $NH_4Cl$-treated *kl/kl*-mice than in wild-type mice, a difference abrogated by LVD. In each, open field, dark-light box, and O-maze $NH_4Cl$-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 $NH_4Cl$ treated *kl/kl*-mice compared to untreated wild-type mice and to *kl/kl*-mice on LVD. In wild-type animals, $NH_4Cl$ treatment did not significantly alter $1,25(OH)_2D_3$, calcium and phosphate concentrations or exploratory behavior. In conclusion, the excessive $1,25(OH)_2D_3$ 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 brain[1]. The extracellular domain of the transmembrane protein may be cleaved off and enter blood or cerebrospinal fluid[1]. Klotho is a powerful inhibitor of 1α-25-hydroxyvitamin D hydroxylase (1 α hydroxylase) thus preventing 1,25-dihydroxyvitamin $D_3$ ($1,25(OH)_2D_3$) formation[1]. Klotho influences mineral metabolism in addition by up-regulation of $Ca^{2+}$ channels[2] and down-regulation of phosphate transport[3,4]. Klotho affects further channels and transport proteins including $Na^+/K^+$-ATPase[5,6], $Na^+/Ca^{2+}$-exchanger[7], $Ca^{2+}$ channels[8], $K^+$ 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 mice[19]. 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 myelination[27] and klotho has been postulated to counteract neurodegeneration[28]. Overexpression of klotho has been shown to enhance cognition[23]. 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 $1,25(OH)_2D_3$ on cerebral function. $1,25(OH)_2D_3$ has previously been shown to affect behavior[30,31], emotions and anxiety[32]. 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 behavior[36]. 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 nigra[46]. VDR

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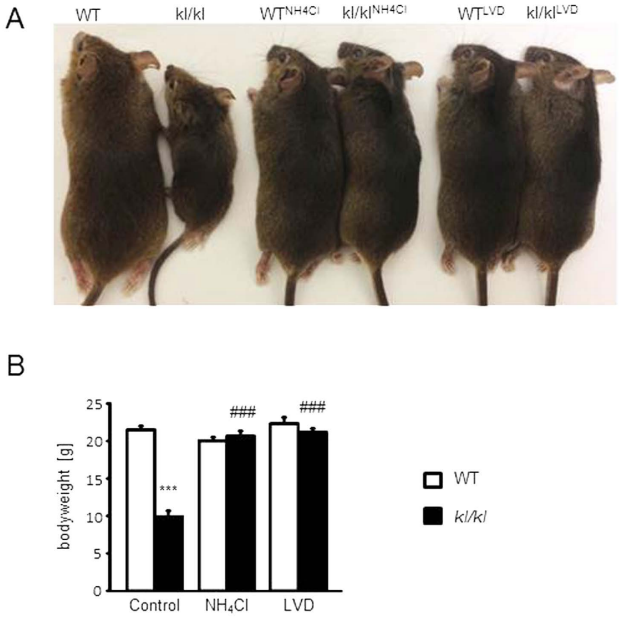


**Figure 1. Effect of $NH_4Cl$ treatment and low vitamin D diet on body weight of wild-type mice and of kl/kl mice.** (**A**) Photograph of male wild-type mice (*WT*) as well as male klotho-hypomorphic mice (*kl/kl*) without (left) or with $NH_4Cl$ treatment (15 g/l in drinking water) without ($NH_4Cl$, middle) and with (LVD, right) additional low vitamin D diet. (**B**) Arithmetic means ± SEM of body weight (n = 12–30) of wild-type (WT, white bars) and *kl/kl* mice (*kl/kl*, black bars) either untreated (left bars, Control), treated with $NH_4Cl$ solution (280 mM in drinking water) ($NH_4Cl$, middle bars) or treated with $NH_4Cl$ 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 symptoms[47]. $1,25(OH)_2D_3$ serum concentration correlates with extraversion[49], 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 $1,25(OH)_2D_3$ formation have been associated with seasonal affective disorders[51–53].

The excessive formation of $1,25(OH)_2D_3$ 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 $NH_4Cl$ into the drinking water fully prevents the severe vascular calcification and rapid ageing of *kl/kl* mice without affecting the excessive formation of $1,25(OH)_2D_3$ and the increase of plasma phosphate and calcium concentrations[54]. $NH_4Cl$ is apparently effective by alkalinizing acidic cellular compartments which compromizes the maturation of TGFß, a critical mediator of osteogenic signaling[54]. Aging and life span are almost identical in $NH_4Cl$ treated *kl/kl*-mice and wild type mice[54]. The $NH_4Cl$ treated *kl/kl* mice would thus be an ideal model to study the effect of excessive $1,25(OH)_2D_3$ on behavior. Thus, *kl/kl* mice and wild-type mice were treated with $NH_4Cl$ (280 mM in drinking water) and with either control diet or vitamin D deficient diet, which has previously been shown to normalize plasma $1,25(OH)_2D_3$ levels in *kl/kl* mice[55]. The behavior of those mice was explored utilizing open field, dark-light box, O-maze, and forced swimming test.

## Results

Without $NH_4Cl$ 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). $NH_4Cl$ treatment increased significantly the body weight of *kl/kl* mice to similar values as the body weight of wild-type mice (Fig. 1B).

Plasma $1,25(OH)_2D_3$ (Fig. 2A), phosphate (Fig. 2B) and $Ca^{2+}$ (Fig. 2C) concentrations were significantly higher in untreated *kl/kl* mice than in wild-type mice, differences not significantly affected by $NH_4Cl$ 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. $NH_4Cl$ 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 $NH_4Cl$ treated *kl/kl* mice than in the respective wild type mice, differences, however, not reaching statistical significance (Fig. 3B)

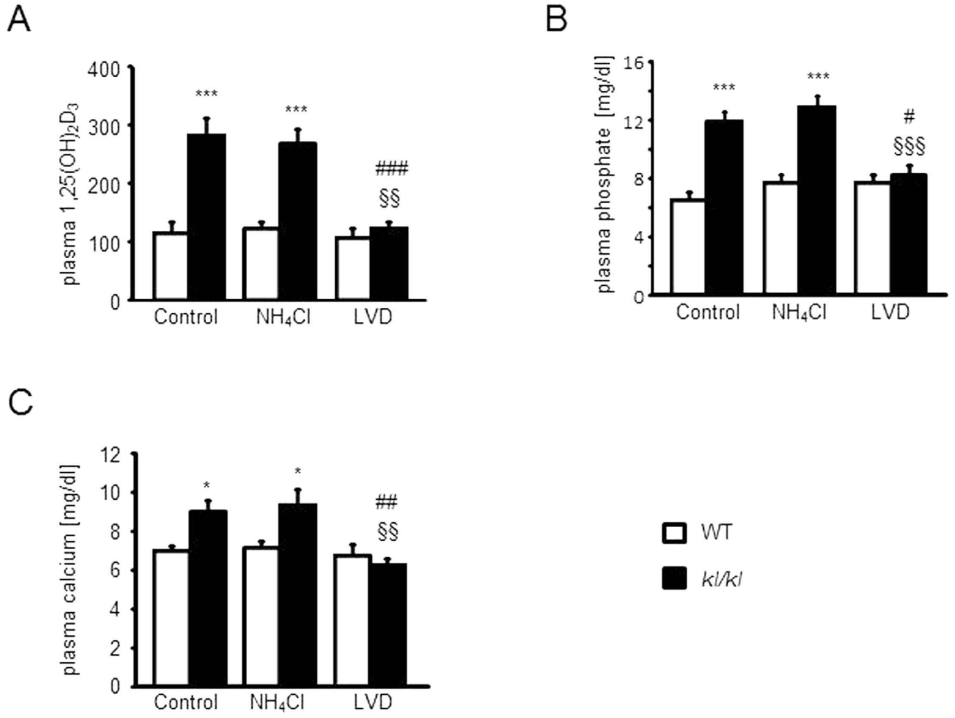


**Figure 2. Effect of $NH_4Cl$ treatment and low vitamin D diet on plasma $1,25(OH)_2D_3$, phosphate, and $Ca^{2+}$ concentrations of wild-type mice and kl/kl mice.** (**A–C**) Arithmetic means ± SEM of (**A**) plasma $1,25(OH)_2D_3$ (n = 6), (**B**) phosphate (n = 12), and (**C**) $Ca^{2+}$ (n = 12) concentrations of wild-type mice (WT, white bars) and *kl/kl* mice (black bars) either untreated, treated with $NH_4Cl$ solution (280 mM in drinking water) or treated with $NH_4Cl$ 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 $NH_4Cl$ treated mice on control diet. (ANOVA).

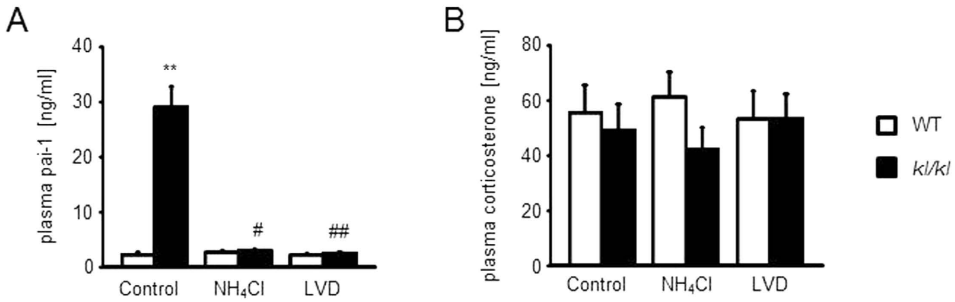


**Figure 3. Effect of $NH_4Cl$ treatment and low vitamin D diet on plasma pai-1and corticosterone levels.** (**A**) Arithmetic means ± SEM (n = 8, ♂ = 4, ♀ = 4 ) of plasma pai-1 concentrations in wild-type mice (WT, white bars) and *kl/kl* mice (black bars) either untreated, treated with $NH_4Cl$ solution (280 mM in drinking water) or treated with $NH_4Cl$ 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 $NH_4Cl$ treated *kl/kl* mice on control diet. (ANOVA).(**B**) Arithmetic means ± 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 $NH_4Cl$ solution (280 mM in drinking water) or treated with $NH_4Cl$ 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), $NH_4Cl$ treated WT mice and $NH_4Cl$ treated *kl/kl* mice ($NH_4Cl$) under regular diet as well as WT mice and *kl/kl* mice under a vitamin D deficient diet (LVD).

In the open-field, $NH_4Cl$ 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 $NH_4Cl$ treated *kl/kl* mice also spent significantly less time

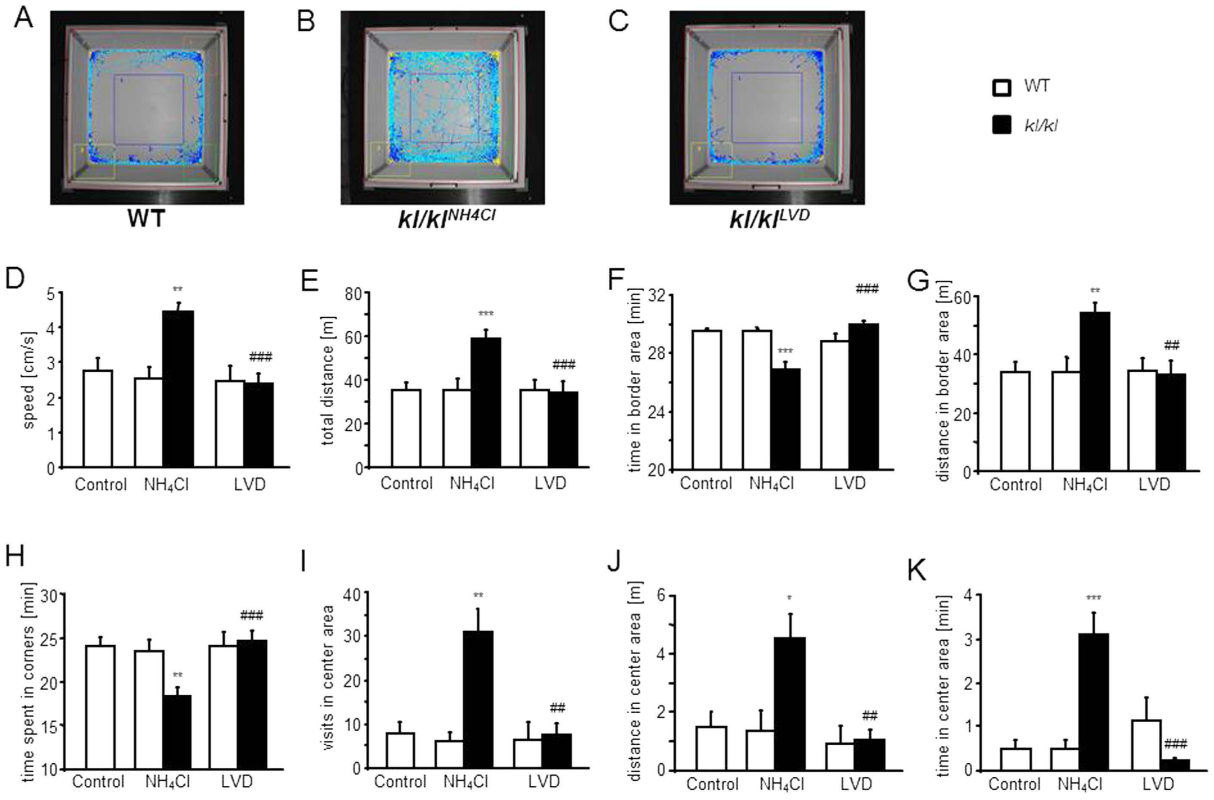


**Figure 4. Effect of $NH_4Cl$ treatment and low vitamin D diet on performance in Open Field Test.** (**A–C**) Photographs of the Open Field arena with representative tracings of an untreated, male wild-type mouse (WT) (**A**), a male, klotho-hypomorphic mouse (*kl/kl*) treated with 280 mM $NH_4Cl$ solution (**B**) and a male, $NH_4Cl$ treated *kl/kl* mouse under vitamin D deficient diet (**C**). (**D–K**) Arithmetic means ± SEM (n = 12–30) 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 | $WT^{NH4Cl}$ | $kl/kl^{NH4Cl}$ | $WT^{LVD}$ | $kl/kl^{LVD}$ | statistics |
|---|---|---|---|---|---|---|
| number of rearings in border area | 84.18 ± 14.05 | 68.87 ± 15.82 | 138.53 ± 10.70 | 60.17 ± 13.66 | 78.10 ± 11.13 | P < 0.0001 ANOVA |
| rearing time in border area [min] | 3.03 ± 0.60 | 2.74 ± 0.68 | 6.37 ± 0.63 | 2.56 ± 0.76 | 2.82 ± 0.50 | P < 0.0001 ANOVA |
| number of rearings in center area | 1.36 ± 0.50 | 1.07 ± 0.93 | 7.67 ± 1.65 | 1.42 ± 0.74 | 0.81 ± 0.45 | P < 0.0001 nonparametric ANOVA |
| Rearing time in center area [s] | 1.23 ± 0.48 | 0.36 ± 0.25 | 10.88 ± 3.01 | 0.18 ± 0.12 | 0.65 ± 0.42 | P < 0.0001 nonparametric ANOVA |

**Table 1. Synopsis of rearing parameters in the open field test (arithmetic means ± 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

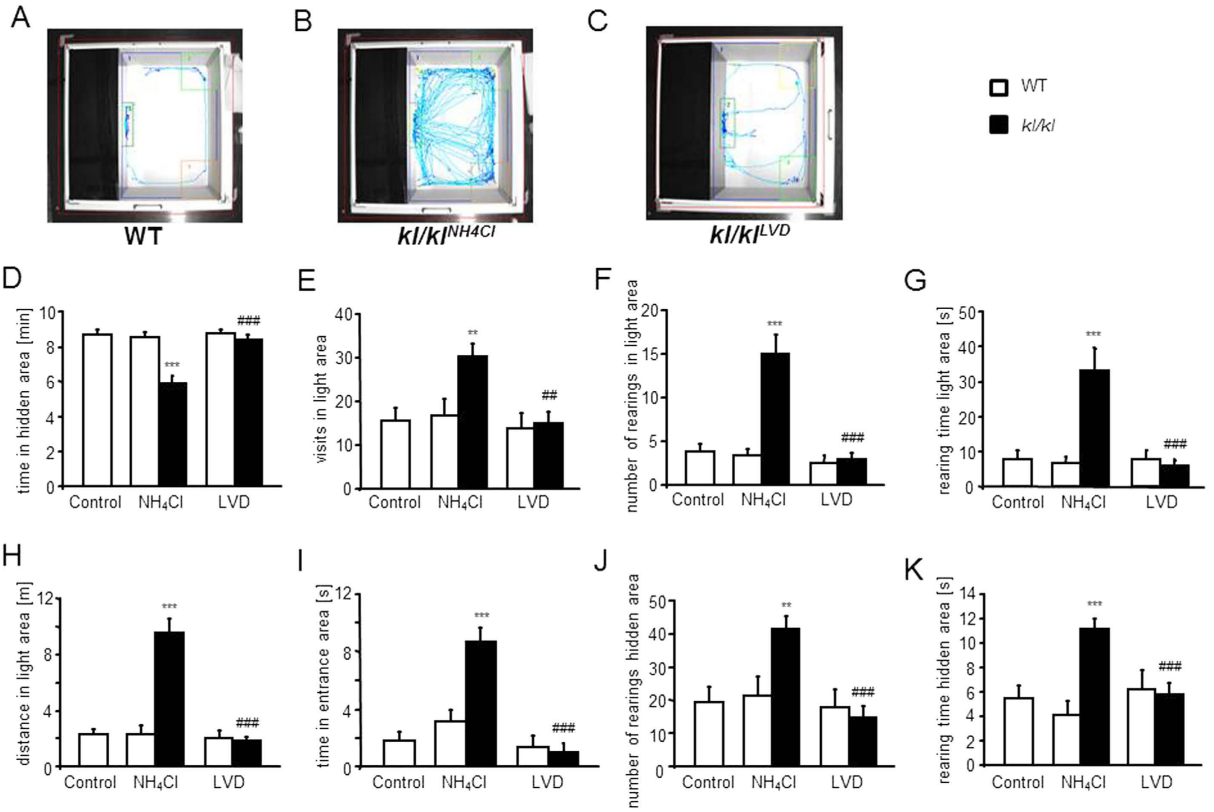


**Figure 5. Effect of $NH_4Cl$ treatment and low vitamin D diet on performance in Light Dark Box.** (**A–C**) Photograph of the Light Dark Box with representative tracings of an untreated male wild-type mouse (WT) (**A**), a male, klotho-hypomorphic mouse (*kl/kl*) treated with 280 mM $NH_4Cl$ solution (**B**) and a male, $NH_4Cl$ treated *kl/kl* mouse under vitamin D deficient diet (**C**). (**D–K**) Arithmetic means ± 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 $NH_4Cl$ solution ($NH_4Cl$, middle bars) or treated with $NH_4Cl$ 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 $NH_4Cl$ treated kl/kl mice. (ANOVA).

like wild-type mice. Again neither $NH_4Cl$ 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 $NH_4Cl$ treated *kl/kl* mice (Fig. 6A–C). They showed significantly more protected and unprotected headdips than wild-type mice (Fig. 6D,E). $NH_4Cl$ 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). $NH_4Cl$ 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 $NH_4Cl$ treated *kl/kl* mice (Fig. 6K) as compared to wild-type mice. Treatment with LDV abrogated the abnormal behavioral phenotype of *kl/kl* mice. In the O-Maze test neither $NH_4Cl$ 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 $NH_4Cl$ 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 $NH_4Cl$ treated *kl/kl* mice and $NH_4Cl$ 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 $1,25(OH)_2D_3$ formation in *kl/kl* mice accounted for

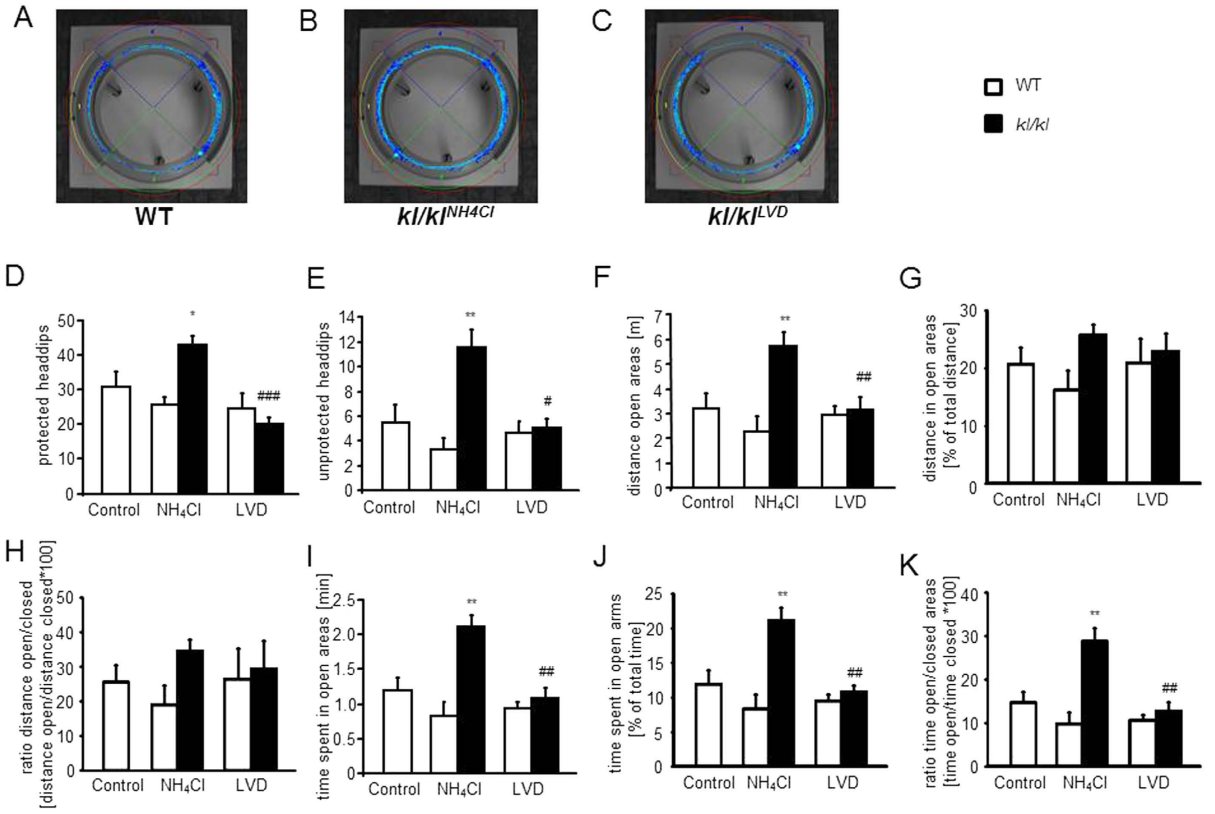


**Figure 6. Effect of $NH_4Cl$ treatment and low vitamin D diet on performance in O-Maze.** (**A–C**) Photograph of the O-Maze with representative tracings of an untreated, male wild-type mouse (WT) (**A**), a male klotho-hypomorphic mouse (*kl/kl*) treated with 280 mM $NH_4Cl$ solution (**B**) and a male, $NH_4Cl$ treated *kl/kl* mouse under vitamin D deficient diet (**C**). (**D–K**) Arithmetic means ± SEM (n = 12–30) 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 | $WT^{NH4Cl}$ | $kl/kl^{NH4Cl}$ | $WT^{LVD}$ | $kl/kl^{LVD}$ | statistics |
|---|---|---|---|---|---|---|
| time spent in light area [min] | 1.28 ± 0.26 | 1.45 ± 0.28 | 4.08 ± 0.45 | 1.26 ± 0.25 | 1.57 ± 0.25 | P < 0.0001 nonparametric ANOVA |
| average speed [cm/s] | 2.43 ± 0.29 | 2.45 ± 0.24 | 4.45 ± 0.32 | 2.48 ± 0.27 | 2.37 ± 0.29 | P < 0.0001 nonparametric ANOVA |

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

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

**Table 3. Synopsis of behavioral parameters in the O Maze test (arithmetic means ± 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

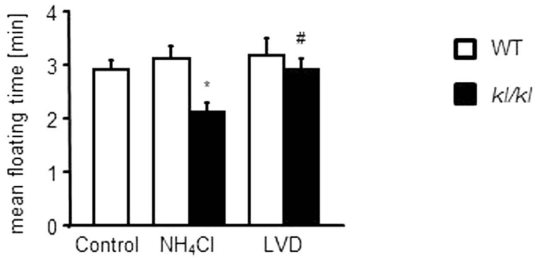


**Figure 7. Effect of $NH_4Cl$ treatment and low vitamin D diet on performance in Forced Swimming Test.** Arithmetic means ± SEM (n = 12–30) of floating time of wild-type mice (WT, white bars) and *kl/kl* mice (*kl/kl* black bars) either untreated (Control, left bar), treated with 280 mM $NH_4Cl$ solution ($NH_4Cl$, middle bars) or treated with $NH_4Cl$ 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 $NH_4Cl$ treated *kl/kl* mice. (ANOVA).

| parameter | | WT | $WT^{NH4Cl}$ | $kl/kl^{NH4Cl}$ | $WT^{LVD}$ | $kl/kl^{LVD}$ |
|---|---|---|---|---|---|---|
| speed [cm/s] | ♂ | 3.00 ± 0.58 | 2.75 ± 0.39 | 4.86 ± 0.47 | 2.37 ± 0.68 | 2.51 ± 0.40 |
| | ♀ | 2.55 ± 0.41 | 2.47 ± 0.58 | 4.11 ± 0.29 | 2.53 ± 0.64 | 2.24 ± 0.45 |
| | ttest | 0.5266 | 0.5381 | 0.1651 | 0.8619 | 0.6612 |
| total distance [m] | ♂ | 37.71 ± 6.95 | 36.03 ± 5.58 | 61.13 ± 6.53 | 35.91 ± 7.44 | 33.81 ± 6.45 |
| | ♀ | 33.09 ± 3.30 | 32.27 ± 9.82 | 56.84 ± 5.37 | 34.74 ± 6.83 | 35.34 ± 7.33 |
| | ttest | 0.5553 | 0.9000 | 0.6128 | 0.9103 | 0.8762 |
| time in border area [min] | ♂ | 29.66 ± 0.10 | 29.48 ± 0.40 | 27.47 ± 0.75 | 29.28 ± 0.32 | 29.86 ± 0.05 |
| | ♀ | 29.35 ± 0.39 | 29.62 ± 0.20 | 26.43 ± 0.66 | 28.45 ± 0.99 | 29.71 ± 0.15 |
| | ttest | 0.4611 | 0.8112 | 0.3062 | 0.4423 | 0.3597 |
| distance in border area [m] | ♂ | 36.31 ± 6.39 | 34.29 ± 5.93 | 58.04 ± 5.78 | 34.40 ± 4.56 | 33.03 ± 6.28 |
| | ♀ | 34.50 ± 3.45 | 31.39 ± 9.61 | 51.21 ± 4.70 | 34.40 ± 6.77 | 34.04 ± 6.77 |
| | ttest | 0.5153 | 0.9585 | 0.3627 | 1 | 0.9141 |
| time spent in corners [min] | ♂ | 24.03 ± 1.62 | 22.33 ± 2.05 | 18.96 ± 0.88 | 23.24 ± 2.32 | 25.41 ± 1.12 |
| | ♀ | 23.97 ± 1.58 | 24.16 ± 1.61 | 17.94 ± 1.62 | 24.91 ± 2.39 | 23.95 ± 2.13 |
| | ttest | 0.9805 | 0.3798 | 0.6167 | 0.6276 | 0.5417 |
| distance in center area [m] | ♂ | 1.48 ± 0.69 | 1.74 ± 1.28 | 5.63 ± 1.32 | 1.35 ± 1.26 | 0.77 ± 0.39 |
| | ♀ | 1.60 ± 0.86 | 0.87 ± 0.48 | 3.09 ± 0.85 | 0.49 ± 0.17 | 1.30 ± 0.68 |
| | ttest | 0.8627 | 0.5802 | 0.1446 | 0.5154 | 0.4969 |
| visits in center area | ♂ | 5.73 ± 1.75 | 5.13 ± 2.75 | 23.39 ± 6.39 | 9.67 ± 7.97 | 5.82 ± 2.68 |
| | ♀ | 10.18 ± 4.68 | 7.25 ± 3.44 | 36.53 ± 8.42 | 3.00 ± 1.75 | 9.60 ± 4.74 |
| | ttest | 0.3837 | 0.6284 | 0.2482 | 0.4329 | 0.4854 |
| time in center area [min] | ♂ | 0.35 ± 0.10 | 0.53 ± 0.40 | 2.53 ± 0.75 | 0.72 ± 0.32 | 0.14 ± 0.05 |
| | ♀ | 0.65 ± 0.39 | 0.39 ± 0.20 | 3.57 ± 0.66 | 1.56 ± 0.99 | 0.29 ± 0.15 |
| | ttest | 0.4611 | 0.8112 | 0.3062 | 0.4423 | 0.3597 |
| number of rearings in border area | ♂ | 96.73 ± 19.95 | 68.50 ± 15.56 | 154.69 ± 15.97 | 68.50 ± 24.14 | 86.82 ± 15.65 |
| | ♀ | 71.64 ± 20.00 | 69.75 ± 30.48 | 126.18 ± 14.07 | 51.83 ± 14.50 | 68.50 ± 16.09 |
| | ttest | 0.3850 | 0.9813 | 0.1916 | 0.5671 | 0.4251 |
| rearing time in border area [min] | ♂ | 3.22 ± 0.81 | 3.18 ± 1.00 | 7.56 ± 0.91 | 2.59 ± 1.14 | 3.10 ± 0.67 |
| | ♀ | 2.83 ± 0.91 | 2.33 ± 0.96 | 6.37 ± 0.63 | 2.56 ± 1.13 | 2.52 ± 0.79 |
| | ttest | 0.7506 | 0.5128 | 0.1016 | 0.9865 | 0.5800 |
| number of rearings in center area | ♂ | 1.46 ± 0.78 | 1.75 ± 1.05 | 5.69 ± 1.67 | 1.67 ± 1.31 | 0.27 ± 0.20 |
| | ♀ | 1.27 ± 0.68 | 0.25 ± 0.29 | 9.18 ± 2.59 | 1.17 ± 0.83 | 1.4 ± 0.95 |
| | ttest | 0.8618 | 0.4543 | 0.3019 | 0.7538 | 0.2364 |
| rearing time in center area [min] | ♂ | 1.36 ± 0.63 | 0.28 ± 0.20 | 6.92 ± 2.05 | 0.18 ± 0.16 | 0.34 ± 0.23 |
| | ♀ | 1.10 ± 0.76 | 0.40 ± 0.36 | 13.91 ± 5.03 | 0.18 ± 0.17 | 0.99 ± 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 ± 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 $1,25(OH)_2D_3$. The effects are,however, not necessarily due to a direct effect of $1,25(OH)_2D_3$ on neuronal function and behavior.

$NH_4Cl$ treatment had no significant effect in wildtype mice indicating that the $NH_4Cl$ treatment does not alter any of the measured parameters on its own. Similar to earlier observations[54], $NH_4Cl$ treatment did not appreciably influence plasma $1,25(OH)_2D_3$, $Ca^{2+}$ and phosphate concentrations. $NH_4Cl$ interferes with osteogenic signaling thus preventing the disastrous tissue calcification in *kl/kl* mice[54].

The present observations underscore the powerful direct or indirect influence of $1,25(OH)_2D_3$ on the brain, which presumably accounts for the various cerebral effects of vitamin D deficiency. Decreased serum levels of the $1,25(OH)_2D_3$ precursor $25(OH)D_3$ 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 extraversion[58]. Conversely vitamin D supplementation decreases the risk to develop psychotic-like symptoms[44].

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 $1,25(OH)_2D_3$, including antioxidant effects, inhibition of inflammation and vascular injury, stimulation of neurotrophins and improvement of metabolic and cardiovascular function[30]. Vitamin D deficiency has been suggested to modify cellular development, dopamine metabolism, and brain morphology[59]. In theory, $1,25(OH)_2D_3$ could affect neuronal function by influencing neuronal or glial cytosolic $Ca^{2+}$ activity[60–62]. $1,25(OH)_2D_3$ may interfere with the cerebral action of glucocorticoids, which are involved in the development of major depression[63]. $1,25(OH)_2D_3$ dependent calcium binding protein has been observed in nuclei influencing the pineal gland[64] and vitamin $D_3$ deficiency may contribute to the desynchronisation in seasonal affective disorders[65].

In wild type animals, dietary vitamin D does not necessarily influence $1,25(OH)_2D_3$ concentration, as $1\alpha$-25-hydroxyvitamin D hydroxylase and thus $1,25(OH)_2D_3$ formation is under tight regulation by FGF23 and klotho[1]. Both, FGF23 and klotho expression are stimulated by $1,25(OH)_2D_3$ and thus $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ is a function of dietary vitamin D even at excessive $1,25(OH)_2D_3$

| 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 $1,25(OH)_2D_3$ formation up-regulated by dehydration[68] and parathyroid hormone[69], FGF23 is up-regulated and $1,25(OH)_2D_3$ formation downregulated by lithium[70,71] and 1α-25-hydroxyvitamin D hydroxylase inhibited by CO-releasing molecule CORM-2[72].

In conclusion, the present observations reveal that disruption of klotho dependent inhibition of 1α-25-hydroxyvitamin D hydroxylase and thus excessive $1,25(OH)_2D_3$ 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) mice[54]. 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 ± 2 °C and the humidity was 55 ± 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 $1,25(OH)_2$-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 detail[73].

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 the same corner of the illuminated compartment and observed for 10 min[74].

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 recorded[76].

**Statistics.** Data are provided as means ± 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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