1,25(OH)\(_2\)D\(_3\) 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)\(_2\)D\(_3\) formation. Klotho-deficient mice (\(kl/kl\)) suffer from excessive plasma 1,25(OH)\(_2\)D\(_3\), Ca\(^{2+}\) and phosphate-concentrations, leading to severe soft tissue calcification and accelerated aging. NH\(_4\)Cl treatment prevents tissue calcification and premature ageing without affecting 1,25(OH)\(_2\)D\(_3\) formation. The present study explored the impact of excessive 1,25(OH)\(_2\)D\(_3\) formation in NH\(_4\)Cl-treated \(kl/kl\)-mice on behavior. To this end \(kl/kl\)-mice and wild-type mice were treated with NH\(_4\)Cl and either control diet or vitamin D deficient diet (LVD). As a result, plasma 1,25(OH)\(_2\)D\(_3\), Ca\(^{2+}\) and phosphate-concentrations were significantly higher in untreated and in NH\(_4\)Cl-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\(_4\)Cl-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\(_4\)Cl treated \(kl/kl\)-mice compared to untreated wild-type mice and to \(kl/kl\)-mice on LVD. In wild-type animals, NH\(_4\)Cl treatment did not significantly alter 1,25(OH)\(_2\)D\(_3\), calcium and phosphate concentrations or exploratory behavior. In conclusion, the excessive 1,25(OH)\(_2\)D\(_3\) 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-dihydroxyvitamin D hydroxylase (1α hydroxylase) thus preventing 1α,25-dihydroxyvitamin D\(_3\) (1,25(OH)\(_2\)D\(_3\)) 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\(^25\). 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)\(_2\)D\(_3\) on cerebral function. 1,25(OH)\(_2\)D\(_3\) has previously been shown to affect behavior\(^30,31\), emotions and anxiety\(^33\). 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\(^37–42\). In humans vitamin D deficiency predisposes to several psychiatric disorders, such as depression, bipolar disorder and schizophrenia\(^35,43–45\). The vitamin D receptor (VDR) and vitamin D metabolizing enzymes are widely expressed in cerebral structures including prefrontal cortex, hippocampus, cingulate gyrus, thalamus, hypothalamus, and substantia nigra\(^46\). VDR

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gene variants are associated with altered behavior\(^{47,48}\) as well as susceptibility to age-related changes in cognitive function and depressive symptoms\(^{47}\). \(1,25\text{(OH)}_2\text{D}_3\) 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 \(1,25\text{(OH)}_2\text{D}_3\) formation have been associated with seasonal affective disorders\(^{51–53}\).

The excessive formation of \(1,25\text{(OH)}_2\text{D}_3\) 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 \(\text{NH}_4\text{Cl}\) into the drinking water fully prevents the severe vascular calcification and rapid ageing of \(kl/kl\) mice without affecting the excessive formation of \(1,25\text{(OH)}_2\text{D}_3\) and the increase of plasma phosphate and calcium concentrations\(^{54}\). \(\text{NH}_4\text{Cl}\) 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 \(\text{NH}_4\text{Cl}\) treated \(kl/kl\)-mice and wild type mice\(^{54}\). The \(\text{NH}_4\text{Cl}\) treated \(kl/kl\) mice would thus be an ideal model to study the effect of excessive \(1,25\text{(OH)}_2\text{D}_3\) on behavior.

Thus, \(kl/kl\) mice and wild-type mice were treated with \(\text{NH}_4\text{Cl}\) (280 mM in drinking water) and with either control diet or vitamin D deficient diet, which has previously been shown to normalize plasma \(1,25\text{(OH)}_2\text{D}_3\) 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 \(\text{NH}_4\text{Cl}\) 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). \(\text{NH}_4\text{Cl}\) treatment increased significantly the body weight of \(kl/kl\) mice to similar values as the body weight of wild-type mice (Fig. 1B).

Plasma \(1,25\text{(OH)}_2\text{D}_3\) (Fig. 2A), phosphate (Fig. 2B) and \(\text{Ca}^{2+}\) (Fig. 2C) concentrations were significantly higher in untreated \(kl/kl\) mice than in wild-type mice, differences not significantly affected by \(\text{NH}_4\text{Cl}\) 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. \(\text{NH}_4\text{Cl}\) 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 \(\text{NH}_4\text{Cl}\) treated \(kl/kl\) mice than in the respective wild type mice, differences, however, not reaching statistical significance (Fig. 3B).
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...
in the border area (Fig. 4F) but still travelled larger distances there (Fig. 4G) than wild-type mice. NH₄Cl 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 NH₄Cl drinking solution or LVD. Rearing behavior is shown in Table 1.

The increased activity of NH₄Cl treated kl/kl mice was also apparent in the light dark transition test (Fig. 5A–C). NH₄Cl 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 NH₄Cl 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 4. Effect of NH₄Cl 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 NH₄Cl solution (B) and a male, NH₄Cl 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 NH₄Cl solution (NH₄Cl, middle bars) or treated with NH₄Cl 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); and ***(p < 0.001) indicates statistically significant differences from NH₄Cl treated kl/kl mice on control diet. (ANOVA).

Table 1. Synopsis of rearing parameters in the open field test (arithmetic means ± SEM).
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 headdips 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 LDV abrogated the abnormal behavioral phenotype of kl/kl mice. In the O-Maze test neither NH4Cl treatment nor LVD had an influence on 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...
the observed differences between NH₄Cl 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
Table 4. Differences between male and female mice in the open field test (arithmetic means ± SEM).

| Parameter                              | Male WT | Male WT+NH4Cl | Male kl/kl | Female WT | Female WT+NH4Cl | Female kl/kl |
|----------------------------------------|---------|---------------|------------|-----------|----------------|--------------|
| 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 |
| t-test                                 | 0.5266   | 0.5381       | 0.1651     | 0.8619    | 0.6612         |
| Total distance (m)                      | 37.71 ± 0.95 | 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 |
| t-test                                 | 0.5555   | 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 |
| t-test                                 | 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 | 34.06 ± 6.77 | 34.04 ± 6.77 |
| t-test                                 | 0.5153   | 0.9585       | 0.3627     | 0.9141    | 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 |
| t-test                                 | 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   |
| t-test                                 | 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   |
| t-test                                 | 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   |
| t-test                                 | 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 ± 16.99 |
| t-test                                 | 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   |
| t-test                                 | 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    |
| t-test                                 | 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   |
| t-test                                 | 0.7945   | 0.7266       | 0.2573     | 1         | 0.4501         |
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In theory, 1,25(OH)2D3 could affect neuronal function by influencing neuronal or glial cytosolic Ca2+. The effects are, however, not necessarily due to a direct effect of 1,25(OH)2D3 on neuronal function and behavior.

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

| Parameter                         | WT   | WTkl/kl | kl/klWT | kl/klkl/kl |
|-----------------------------------|------|---------|---------|------------|
| time in hidden area [min]         | 6.7 ± 0.48 | 6.81 ± 0.29 | 6.26 ± 0.57 | 6.8 ± 0.38 |
| visits in light area              | 6.1 ± 0.23 | 6.87 ± 0.53 | 5.66 ± 0.67 | 6.91 ± 0.35 |
| time in entrance area [min]       | 0.82 ± 0.065 | 0.5645 | 0.5234 | 0.5208 |
| number of rearings in light area  | 5.55 ± 1.06 | 3.63 ± 1.43 | 3.50 ± 1.63 | 3.00 ± 0.62 |
| rearing time light area [s]       | 4.27 ± 1.18 | 3.00 ± 1.07 | 3.14 ± 2.85 | 1.67 ± 0.72 |
| time in light area [min]          | 0.65 ± 0.16 | 0.7377 | 0.3628 | 0.9444 |
| distance in light area [m]        | 6.89 ± 1.40 | 5.37 ± 2.14 | 33.31 ± 6.41 | 5.66 ± 2.86 |
| number of rearings in hidden area | 7.20 ± 2.33 | 8.51 ± 2.45 | 33.32 ± 11.67 | 6.06 ± 2.41 |
| rearing time hidden area [s]      | 0.7673 | 0.3493 | 0.9985 | 0.9166 |
| time in light area [min]          | 2.51 ± 0.55 | 2.27 ± 0.77 | 8.87 ± 1.05 | 1.77 ± 0.57 |
| speed [cm/s]                      | 2.17 ± 0.39 | 2.33 ± 1.09 | 10.27 ± 1.58 | 2.33 ± 0.96 |

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 observations44, 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 mice44.

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 1,25(OH)2D3 are observed in patients suffering from depression66,67. Conversely, vitamin D supplementation has been reported to counteract depressive symptoms51–53. 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 function66. 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+ activity60–62. 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 gland44 and vitamin D 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 concentrations, as 1,α,25-hydroxyvitamin D hydroxylase and thus 1,25(OH)2D3 formation is under tight regulation by FGF23 and klotho4. Both, FGF23 and klotho expression are stimulated by 1,25(OH)2D3 and thus 1,25(OH)2D3 formation is limited by negative feedback regulation144.66,67. 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.
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)2D3 formation up-regulated by dehydration61 and parathyroid hormone62. FGF23 is up-regulated and 1,25(OH)2D3 formation downregulated by lithium70,71 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(OH)2D3 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-1 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 inserional 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/HeJ. 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) mice64. The animals were housed

| parameter                           | WT         | WT+NH4Cl   | A/K+NH4Cl | WT+CORM | A/K+CORM |
|-------------------------------------|------------|------------|-----------|---------|----------|
| protected headips                   | ♂ 27.00±6.15 | 23.88±2.86 | 45.65±3.65 | 22.83±5.06 | 20.50±2.66 |
|                                     | ♀ 34.27±6.42 | 29.73±3.76 | 41.00±3.77 | 28.50±7.21 | 19.82±2.93 |
| ttest                               | 0.4231     | 0.4412     | 0.4735     | 0.6858   | 0.8660   |
| unprotected headips                 | ♂ 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 | 36.64±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).
had access to either tap water or a solution of NH₄Cl in tap water (280 mM) ad libitum and were fed either
day and the home cage rack was brought to the test room at least 30 min before each experiment and dry sur-
faces 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 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 D₃ (IDS,
Boldsom, 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[23].
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[4].
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[23,27]. 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[26].

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

| parameter                  | WT   | WT NH4Cl | kl/kl NH4Cl | WT LVD | kl/kl LVD |
|----------------------------|------|----------|-------------|--------|-----------|
| mean floating time [min]   | 3.10 | 3.01     | 2.20        | 2.93   | 2.81      |

### Table 8. Number of animals used in the experiment.

|                       | 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           |
Statistics. Data were 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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