Complexin II is essential for normal neurological function in mice

Dervila Glynn, Rachel A. Bortnick and A. Jennifer Morton*

Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1PD, UK

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Complexins (CPLXs) are modulators of synaptic vesicle release. At 1 year of age, CPLXII knockout (KO) mice appear normal. However, behavioral testing reveals underlying deficits of motor and cognitive function in these mice. We found motor deficits on the rotarod, and learning deficits in the Morris water maze (both acquisition and reversal) and the two-choice swim tank (reversal). The reversal learning deficits are particularly noticeable, being present from the earliest time of testing, when most other behaviors are normal. CPLXII KO mice also fail to develop adult patterns of exploratory behavior in the open field and show deficits in interactive grooming behaviors. The behavioral deficits worsen with age. For example, while rotarod performance is normal until 10 weeks, it is impaired from 24 weeks onwards. Similarly, deficits in spatial learning in the Morris water maze are mild at 8 weeks, but pronounced by 1 year of age. The deficits seen in CPLXII KO mice are not due to physical weakness, since their ability to run, swim and grip is unimpaired. Rather, the mice appear to have deficits of higher function. The deficits seen in CPLXII KO mice are strikingly similar to those seen in the R6/2 model of Huntington’s disease (HD) where a progressive depletion of CPLXII is seen. This suggests that depletion of CPLXII contributes to cognitive abnormalities in R6/2 mice. Given that decreased expression of CPLXII is seen in HD and schizophrenic patients, a role for CPLXII depletion should be considered in other diseases where motor, cognitive and psychiatric symptoms co-exist.

INTRODUCTION

Complexin II are small (18–19 kDa) cytoplasmic proteins that modulate exocytosis of neurotransmitter via an interaction with the SNARE complex (1–3). They exist as two major isoforms in the brain, CPLXI and CPLXII (4). CPLXs play an essential role in the brain, since double knockout (KO) mice die at birth (5). However, when one or other isoform of CPLX is knocked out, the mice survive to maturity (5,6), suggesting that they each play important but complementary roles. The two isoforms of CPLX are differentially distributed in the brain (7). As might be expected, the phenotypes of the single KOs are very different (5). However, whereas the CPLXI KO mouse exhibits severe neurological symptoms characterized by ataxia, the CPLXII KO mouse appears normal (5). This is surprising, given that CPLXII expression is altered in several different neurological diseases, including Huntington’s disease (HD) (8) and schizophrenia (9–11). We have previously suggested a role for CPLXII in neurological dysfunction in HD, since, as well as being reduced in human HD brain, CPLXII protein is selectively depleted in an animal model of HD (R6/2 line) (12). Further, abnormalities in hippocampal long-term potentiation (LTP) are present in both R6/2 mice (13) and CPLXII KO mice (6,14). However, our suggestion is confounded by the fact that CPLXII KO mice appear to have a normal phenotype (5).

Although superficially problematic, the lack of overt phenotypic abnormalities in CPLXII KO mice does not exclude a role for CPLXII depletion in the HD phenotype. R6/2 mice show an adult-onset, progressive neurological phenotype that profoundly affects their behavior and leads to their premature death (15). Nevertheless, several weeks before R6/2 mice show overt symptoms, significant abnormalities in both motor and cognitive function can be detected by behavioral testing (16,17). In other mouse models too, neuronal pathology and behavioral deficits are present in mice that are phenotypically ‘normal’ (18–20). Finally, in HD patients themselves, neurological impairments can be measured several years before overt symptoms appear (for references see 21). We wondered if CPLXII KO mice had neurological impairments that were not manifested as overt symptoms. In this study, the behavior of CPLXII KO mice was characterized using a battery of behavioral tests designed to detect both cognitive and motor deficits. We report that despite their ‘normal’ appearance,
CPLXII KO mice have profound abnormalities in behavior that would constitute a disadvantageous phenotype in a normal environment.

RESULTS

CPLXII KO mice have reduced body weights

CPLXII KO mice are physically indistinguishable from wild-type (WT) mice (Fig. 1A and B). However, CPLXII KO mice were significantly lighter than WT mice from weaning onwards (Fig. 1C; genotype, \( F_{1,98} = 25.19, P < 0.001 \)) and remained so throughout the experiment. There were no significant differences in the growth rates (CPLXII KO mice gained 1.033 ± 0.12 g/week while WT mice gained 1.032 ± 0.08 g/week; \( P > 0.05 \), linear regression).

CPLXII KO mice have higher SHIRPA abnormality indices than WT mice

Abnormality indices. The mean abnormality index for CPLXII KO mice was significantly higher than that for WT mice at all ages tested (Fig. 1D, \( P < 0.001 \)). This is particularly revealing since CPLXII KO mice display no overt abnormalities in behavior and, except for the presence of whiskers (see below), are indistinguishable from WT mice.

Body position. Significant differences were found in parameters that reflect body position. At all time points CPLXII KO mice exhibited an increased incidence of ‘abnormal pelvic elevation’, manifested as a lowered pelvis that ‘barely touches’ the surface of the cage floor (\( \chi^2 = 5.379, P < 0.05 \); \( \chi^2 = 13.48, P < 0.001 \); \( \chi^2 = 6.403, P < 0.05 \) for 10, 20 and 30 weeks, respectively). CPLXII KO mice also displayed an abnormal dragging tail position (\( \chi^2 = 4.010, P < 0.05 \); \( \chi^2 = 5.770, P < 0.05 \); \( \chi^2 = 6.734, P < 0.01 \), for 10, 20 and 30 weeks respectively).

Whisker trimming behavior. A significant difference was found in the incidence of whisker trimming (Fig. 1A and B; \( \chi^2 = 60.12 \) at 20 weeks, \( P < 0.001 \)). Whereas all but one of the WT mice (31/32) had been trimmed of most or all of their whiskers by 20 weeks of age, CPLXII KO mice all retained their whiskers, even at 30 weeks of age. This suggests a difference between CPLXII KO mice and WT mice in social interaction.

CPLXII KO mice make fewer attempts to escape from the cage but show normal strength in the hang wire test

CPLXII KO mice made significantly fewer climbing attempts at 16 weeks compared with WT mice (Fig. 1E, \( P < 0.001 \)). (Data for other time points are not shown.) The hang wire test (Fig. 1F) was used to assess neuromuscular strength. Data were collected between 10 and 30 weeks. At all ages, CPLXII KO mice clung to the wire longer than WT mice throughout testing (genotype, \( F_{1,62} = 8.92, P < 0.01 \); age × genotype, \( F_{4,248} = 3.05, P < 0.05 \)). Data from 16 weeks (Fig. 1F) are typical of that collected at other time points (other data are not shown). It is possible that differences in weight affected hang wire performance, since CPLXII KO mice were consistently lighter than WT mice. Analysis revealed that, at 16 weeks, there was a correlation between body weight and hang wire score for CPLXII KO mice, but not for WT mice (Fig. 1G; CPLXII KO—\( y = -3.1051 + 130.24, r^2 = 0.355, P < 0.001 \); WT—\( y = -1.7671 + 79.928, r^2 = 0.0976, P = NS \)). However, CPLXII KO mice are clearly not weaker than WT mice.

Swimming style and strength is normal in CPLXII KO mice

The swim tank was used to investigate motor function and coordination (Fig. 1H). During training, all mice improved significantly with respect to the time they took to mount the platform and swim speed (data not shown). There were no significant differences between the groups at any point during training and no significant interaction of training day × genotype. This suggests that WT and CPLXII KO mice improved their performance of the task at comparable rates. By the time they reached the test stage, mice (both WT and CPLXII KO) swam rapidly toward the escape platform with their front paws tucked up into their chests and using their hind limbs for propulsion. Although there was a trend for CPLXII KO mice to show some difficulty in mounting the platform and to perform the task more slowly (Fig. 1H, middle panel), this did not reach statistical significance. There was no significant difference between WT and CPLXII KO mice in swim speed, latency to mount the platform, or the number of attempts needed to climb onto the escape platform. Furthermore, there was no difference in the number of ‘floaters’ (mice that floated in the tank as opposed to swimming; 1/32 WT compared with 3/32 CPLXII KO), the number of mice with poor direction, and the number of mice making more than one attempt to climb the onto the platform (data not shown). Thus, at 14 weeks, the CPLXII KO mice did not have significant motor deficiencies as measured by swimming ability.

CPLXII KO mice show reduced locomotion and exploration in the open field

Latency to reach the periphery. When placed in the open field for the first time, WT mice moved quickly to the edges of the open field apparatus, whereas CPLXII KO mice took significantly longer to reach the periphery (Fig. 2A; \( P < 0.01 \)). However, after the first day of testing there was no significant difference between groups in latency to reach the periphery.

Total activity. CPLXII KO mice were less active than WT mice (Fig. 2B; \( F_{1,34} = 9.75, P < 0.01 \)). However, with age, while WT mice showed increased total activity in the open field, activity of CPLXII KO mice remained constant. There was a significant interaction between time and genotype (\( F_{6,204} = 2.60, P < 0.05 \)) and by 16 weeks CPLXII KO mice were significantly less active than WT mice (\( P < 0.01 \)).

Percentage time spent in the periphery. Although there was a reduction in the total activity of CPLXII KO mice, the percentage of peripheral squares they entered was similar to WT mice.

Overall, CPLXII KO mice are lighter than WT mice and show abnormalities in behavior and coordination that suggest a difference in motor function.
Figure 1. Health and strength measures of CPLXII KO mice. Photographs of WT (A) and CPLXII KO (B) mice aged 15 months show they are physically indistinguishable from each other, with bright eyes and glossy healthy coats, although the body weight of CPLXII KO mice was consistently lower than that of WT littermates (C). SHIRPA analysis revealed that CPLXII KO mice exhibited higher abnormality indices than WT mice (D) and made fewer cage escape attempts at 16 weeks compared with WT mice (E). However, CPLXII KO mice are not weaker than WT mice in the hangwire test (F). There was a significant correlation between body weight and hang wire score for CPLXII KO mice (G; \( P < 0.001 \)), but not for WT mice. Swimming strength was measured in the swim tank (H). There was no difference between CPLXII KO and WT mice with regard to swim speed, latency to mount platform or the number of attempts needed to climb onto the escape platform. (CPLXII KO mice = open symbols and bars, WT mice = solid symbols and bars). Symbols and bars show the mean±SEM of each group on each measure. Where error bars are not visible, they are obscured by the symbols. Asterisks indicate significant differences between WT control and CPLXII KO mice (\( ** P < 0.01, *** P < 0.001 \)).
in all trials (Fig. 2C). This suggested that there was no difference in levels of anxiety between CPLXII KO and WT mice (22).

Exploration and rearing. Reduced exploratory behavior in CPLXII KO mice was reflected as a decreased incidence of rearing activity in the open field (Fig. 2D–F). CPLXII KO mice reared significantly less frequently than WT mice both with and without wall support in the periphery, and in the center of the open field (F, genotype, F_{1,34} = 38.46, P < 0.001; F_{6,204} = 19.19, P < 0.001; F_{6,204} = 6.27, P < 0.05, respectively). The difference was due not only to a lower level of exploration by the CPLXII KO mice, but also to an increased level of exploratory activity by the WT mice. In particular, the number of times WT mice reared in the center of the field and in the periphery increased with age (age/C2 genotype, F_{6,204} = 4.20, P < 0.05; F_{6,204} = 2.92, P < 0.05; F_{6,204} = 2.44, P < 0.05, respectively). It appears that as WT mice became habituated to the apparatus, they exhibited more exploratory behaviors. In contrast, CPLXII KO mice reared infrequently, and this did not change with time.

Grooming. Overall, CPLXII KO mice performed fewer complete grooming cycles (Fig. 2G) than WT mice (genotype, F_{1,34} = 6.90; P < 0.05). This is consistent with the general reduction in co-ordinated activity observed in CPLXII KO mice.

Urination and fecal boli. There was no main effect of genotype on the number of fecal boli left in the open field (Fig. 2H). There was a significant interaction of age × genotype (F_{6,204} = 6.90; P < 0.001), although the significance of this is not clear. There were no significant differences between WT and CPLXII KO mice (solid symbols and bars). Symbols and bars indicate the mean ± SEM for each measure. Where error bars are not visible, they are obscured by the symbols. Behavior in the open field was analyzed in CPLXII KO mice using a number of measures [latency to reach the periphery (A), total activity (B), and the proportion of peripheral squares entered (C)]. Exploratory behavior was measured by quantifying rearing activity. The number of times the mice rear in the open field with (D) and without wall support (E) and in the center of the open field (F) is shown. Other general parameters that were measured were the incidence of full cycles of grooming (G), defecation (H) and number of urinations (I). Asterisks indicate significant differences between WT and CPLXII KO mice (*P < 0.05, **P < 0.01).
KO mice in the number of urinations (Fig. 2I) left in the open field during the 10 min period. Together these data suggest there is little difference in anxiety between the WT and CPLXII KO mice.

CPLXII KO mice show abnormalities in their gait and stride length

Footprint patterns of CPLXII KO mice and WT controls made at 8 months of age are illustrated in Figure 3A and B. Most WT mice (25/28) walked in a straight line away from the experimenter, with a regular evenly alternating gait. By contrast, nearly half of the CPLXII KO mice (11/26) needed more than one attempt to complete the task (compared with 3/28 for WT mice). Furthermore 9/26 of the CPLXII KO mice scuffled backwards when initially placed in the arena (compared with 3/28 for WT). Of the CPLXII KO mice from which footprints were taken successfully, it is notable that half (13/26) of them weaved from side to side while walking along the runway. The stride length of the male WT mice was significantly longer than that of the female mice ($P < 0.05$), although gender differences were not seen in the CPLXII KO mice. The hind base and front base measurements (Fig. 3C) of CPLXII KO mice were significantly greater than that of WT mice ($P < 0.001$ and $P < 0.01$, respectively). This may account for the lowered body position identified during SHIRPA analysis. In addition, CPLXII KO mice displayed a trend for shorter stride length (Fig. 3D) than WT mice, which reached significance for left-hind stride and right-front stride ($P < 0.01$ for both, unpaired two-tailed $t$-test). These alterations in stride length possibly give rise to the weaving footprint patterns displayed by these mice.

CPLXII KO mice show impaired performance on the visual cliff avoidance task

To assess complex visual discrimination learning, we used the visual cliff avoidance test (Fig. 4A). There was no difference between the WT and CPLXII KO mice in the latency to dismount the platform (Fig. 4B), although a significantly greater number of CPLXII KO mice ventured onto the cliff side immediately upon dismounting the platform (Fig. 4C; $\chi^2 = 7.914$). Moreover, CPLXII KO mice spent a significantly longer amount of time over the cliff (Fig. 4D; $P < 0.01$ using the two-tailed unpaired $t$-test). A similar number of mice in both groups (8/32 WT compared with 7/31 CPLXII KO) failed to dismount the platform during the entire trial, indicating that there was no difference in anxiety or motivation between WT and CPLXII KO groups.
CPLXII KO mice show progressive impairment on the rotarod

Repeated testing of mice on the rotarod over a 9 month time period revealed progressive impairments in the performance of CPLXII KO mice compared with WT mice (Fig. 5A). Young adult mice learned the task and performed normally, with both groups exhibiting comparable performance at all speeds up to 10 weeks of age. However, when mice were retrained and tested at 24 weeks of age, significant differences emerged. WT mice were able to maintain high levels of performance on the lowest four speeds and did not exhibit any significant change in performance at the higher speeds. However, CPLXII KO mice fell off the rotarod significantly sooner than WT mice at all but the lowest speed. At 28 and 40 weeks of age, CPLXII KO mice showed significant deficits at all speeds, not only the fastest (genotype, $F_{1,34} = 21.4$; age, $F_{7,238} = 10.91$; genotype x age, $F_{7,238} = 11.51$). (All speeds were included in the analyses. However, for clarity of presentation, data from 8, 20, and 33 rpm are not included in the figures.) There were some gender effects, with female CPLXII KO mice performing significantly better at 24 rpm than male CPLXII KO mice, and female WT mice performing significantly better at 24, 31 and 44 rpm than male mice. However, the genotype effects were robust, with WT mice performing better than CPLXII KO mice at all speeds, irrespective of their gender.

CPLXII KO mice (group 2) that were naive to the task, were tested on the rotarod for the first time at 24 weeks [an age by which the repeatedly tested CPLXII KO mice (group 1) exhibited significant deficits]. These mice showed no significant differences in performance at 5, 8 or 15 rpm (Fig. 5B). However, the performance of the CPLXII KO mice was significantly worse than that of the WT mice (Fig. 5B) at the five highest speeds (20, 24, 31, 33 and 44 rpm). For clarity, data from 8, 20 and 33 rpm are not included in the figures.

CPLXII KO mice show impairments in the Morris water maze

Acquisition and reversal training. The hidden platform version of the Morris water maze (MWM) was used to test spatial learning at 8 weeks of age. The results of hidden platform training and the probe trials are shown in Figures 6 and 7 respectively.

During initial acquisition training (Fig. 6A), both groups learned to locate the platform. However, CPLXII KO mice took longer to reach the platform over the course of the training and improved more slowly than the WT mice (training day x genotype, $F_{6,198} = 2.27$, $P < 0.05$). Analysis of path-length showed that during acquisition training WT and CPLXII KO mice swam comparable distances to find the hidden platform. However, WT mice improved faster than CPLXII KO mice (training day x genotype, $F_{6,198} = 2.82$, $P < 0.05$). There was a trend for CPLXII KO mice to swim more slowly during acquisition training and for CPLXII KO mice to show an increase in thigmotaxis during acquisition training, but neither
Figure 5. Progressive deficits on rotarod in CPLXII KO mice. WT (solid symbols and bars) and CPLXII KO (open symbols and bars) mice were tested using eight different speeds on an accelerating rotarod (5–44 rpm), five of which are shown here. The means ± SEM of the duration of balance or latency to fall (maximum trial length = 60 s) for two trials at each speed were recorded. (A) Group 1 mice were tested repeatedly between 5 weeks and 10 months. (B) Group 2 mice were trained and tested only at 24 weeks. Symbols and bars indicate the mean ± SEM for latency to fall from the rotarod. Where error bars are not visible, they are obscured by the symbols. Asterisks indicate significant differences between WT and CPLXII KO mice (*P < 0.05, **P < 0.01, ***P < 0.001).
effect reached significance (Fig. 6A, top-right panels). There was no gender effect on swim speed except at the 8 week time point, when male CPLXII KO mice swam slightly faster than female CPLXII KO mice (\(P < 0.05\)).

CPLXII KO mice exhibited significant deficits in the reversal task, taking significantly longer to find the platform on days 10–13 of reversal training (Fig. 6A, \(F_{1,33} = 8.27, P < 0.01\)). CPLXII KO mice also swam further to reach the platform, consistent with the significant difference in latency during reversal training (Fig. 6A; genotype, \(F_{1,33} = 5.47, P < 0.05\); training day × genotype; \(F_{6,198} = 3.55, P < 0.01\)).

**Probe trials.** Probe testing show that at 8 weeks, both WT and CPLXII KO mice learned the location of the hidden platform during acquisition training and also learned the new position during reversal training, since mice in both groups spent the majority of their time in the quadrant previously containing the platform (Fig. 7A). However, the preference for the target quadrant over the other three quadrants was not as robust in the CPLXII KO groups as it was for the WT mice. There was a significant main effect of quadrant during the acquisition probe trial (\(F_{3,99} = 27.42, P < 0.001\)). WT mice showed a strong preference (\(P < 0.01\)) for the training quadrant over all three other quadrants, while CPLXII KO mice showed a strong preference (\(P < 0.01\)) for the training quadrant over the opposite and adjacent left quadrants and a preference (\(P < 0.05\)) for the training quadrant over the adjacent right quadrant.

There was a main effect of quadrant during the reversal trial probe (Fig. 7A; \(F_{3,99} = 26.38, P < 0.001\)). WT mice showed a strong preference (\(P < 0.01\)) for the target quadrant over all three other quadrants, while CPLXII KO mice showed a strong preference (\(P < 0.01\)) for the target quadrant over the opposite and adjacent left quadrants and a preference (\(P < 0.05\)) for the target quadrant over the adjacent right quadrant.

**Figure 6.** Progressive learning deficits in the Morris water maze in CPLXII KO mice. Escape latency, pathlength, swimming speed and thigmotaxis for WT (solid symbols) and CPLXII KO (open symbols) mice during acquisition (days 1–7) and reversal (days 8–14) training at 8–9 weeks (A) and 1 year of age (B). Sample swim paths for acquisition and reversal training in WT and CPLXII KO mice at 1 year are shown in (C). Symbols indicate mean ± SEM on each measure. Where error bars are not visible, they are obscured by the symbols.
preference \((P < 0.01)\) for the training quadrant over the opposite and adjacent left quadrants but spent a similar amount of time in the target quadrant and the adjacent right quadrant.

A more stringent measure of spatial navigation during the probe trial than quadrant preference is obtained by comparing the number of times a mouse crosses the correct location of the target platform with the number of crossings of the three other potential platform positions. Analysis of crossings revealed a main effect of platform position during both acquisition and reversal probe trials at 8–9 weeks (Fig. 7A; \(F_{3,102} = 18.15, P < 0.001\); \(F_{3,102} = 26.26, P < 0.001\)), but no effect of genotype.

Impairments in the Morris water maze worsen with age

MWM testing was repeated at one year. During acquisition training, all CPLXII KO and WT mice learned to find the platform (Fig. 6B), although WT mice improved more quickly than CPLXII KO mice (training day \(\times\) genotype, \(F_{6,192} = 3.47, P < 0.01\)). On the last day of training (day 7), WT mice found the platform in 68/68 trials and CPLXII KO mice found the platform in 65/68 trials \((P > 0.05, \chi^2\) test). The improvement in path-length was faster in WT mice than that it was in the CPLXII KO mice, where improvement was not significant until day 7 (training day \(\times\) genotype, \(F_{6,192} = 12.23, P < 0.01\)). There was no difference between swim speeds of WT and CPLXII KO mice, thus the deficits CPLXII KO mice display during acquisition training cannot be attributed to reduced swim speed. There was no difference in thigmotaxis between WT and CPLXII KO mice (Fig. 6B, right-hand panel).

At 1 year of age, CPLXII KO mice exhibit severe learning impairments during reversal learning. CPLXII KO mice took significantly longer than WT mice to find the platform (Fig. 7B). After reversal training, the probe trial revealed that CPLXII KO mice had not learned the new position of the platform, as they spent a similar amount of time in all four quadrants. CPLXII KO mice showed no preference for the target over equivalent sites in other quadrants.
(Fig. 6B; genotype, \(F_{1,32} = 12.33, P < 0.01\)). On the last day of reversal training (day 7), WT mice found the platform in 67/68 trials whereas CPLXII KO mice found the platform in only 57/68 trials (\(P < 0.01, \chi^2\) test). In addition, CPLXII KO mice swam longer pathlengths (Fig. 6B) to find the platform (\(F_{1,32} = 15.15, P < 0.01\)) and improved more slowly than WT mice (training day \(\times\) genotype, \(F_{6,192} = 3.96, P < 0.01\)). There was no difference in swim speed between CPLXII KO and WT mice during reversal training. CPLXII KO mice did not differ from WT mice with respect to thigmotaxis (Fig. 6B, right-hand panel).

**Probe trials.** The probe trial following acquisition training at 1 year showed that WT mice learned the location of the platform, whereas CPLXII KO mice showed mild impairments at the task (Fig. 7B). Acquisition probe trial analysis revealed a significant main effect of quadrant (\(F_{3,96} = 26.45, P < 0.001\)). WT mice displayed a strong preference (\(P < 0.01\)) for the target quadrant over all other three quadrants, while CPLXII KO mice showed a strong preference (\(P < 0.01\)) for the target quadrant over the opposite and adjacent left quadrants but spent a similar amount of time in the target quadrant and the adjacent right quadrant.

After reversal training, probe testing again revealed a main effect of quadrant (Fig. 7B; \(F_{3,96} = 11.90, P < 0.001\)). WT mice had learned the new location of the platform and showed a strong preference (\(P < 0.01\)) for the target quadrant over all other quadrants. By contrast, CPLXII KO mice had not learned the new position of the platform, and spent a similar amount of time in all four quadrants.

At 1 year of age, there was a main effect of platform position during the acquisition probe trial (Fig. 7B; \(F_{3,96} = 37.05, P < 0.001\)) and no effect of genotype. However, WT mice made significantly more target platform crossings than CPLXII KO mice during the reversal probe trial (platform \(\times\) genotype, \(F_{3,96} = 4.03, P < 0.01\)). CPLXII KO mice showed no preference for crossing the target platform.

The swim paths taken on the seventh day of acquisition or reversal training at 1 year of age suggest that CPLXII KO and WT mice use different strategies for locating the platform. Examples of pathways are shown in Figure 6C. WT mice appear to use the external cues to locate the hidden platform, swimming in straight lines and taking short direct paths. In contrast, CPLXII KO mice display circular swimming patterns. This is the case with both acquisition and reversal learning, although it is more obvious in the reversal pathway shown here, because the mouse did not reach the platform.

The swim paths of the probe trials (Fig. 7C) taken at 1 year also differ between the WT and CPLXII KO mice. The configurations of the swimpaths taken by WT mice during acquisition and reversal probe trials and CPLXII KO mice during acquisition probe trial are similar (although the CPLXII KO mouse does not spend the majority of time in the target quadrant). However, during the reversal probe, CPLXII KO mice continue to swim in circles, and indeed in the example shown, the mouse appears to perseverate on the previous platform location.

**Visible platform.** The profound deficits in the CPLXII KO mice could be due to deficits in behavioral determinants other than spatial learning, such as motor co-ordination, vision or motivation. Therefore we tested the mice in a visible platform version of the MWM test. In contrast to the poor performance in the reversal task, the CPLXII KO mice swam straight to the platform, and there was no difference in the time taken for WT or CPLXII KO mice to find the visible platform (5.0 \(\pm\) 0.8 s compared with 5.4 \(\pm\) 0.7 s; \(P > 0.05\), unpaired two-tailed \(t\)-test). This suggests that the deficits were not due to deficits in motor co-ordination, vision or motivation to find the platform.

**CPLXII KO mice show mild impairments in acquisition learning in the two-choice swim tank, but major impairments in reversal learning**

Acquisition of a simple visual (non-spatial) discrimination task (Fig. 8A) was assessed in CPLXII KO and WT mice aged 30–31 weeks. All mice from both genotypes learned to swim towards the light within 7 days of acquisition training. However, in the first 6 days of training, the mean percentage of correct choices made during the first 10 trials was lower for CPLXII KO mice than WT mice (Fig. 8B; genotype, \(F_{1,29} = 6.93, P = 0.05\)). Moreover, whereas the WT group reached criterion (90% correct choices during the first 10 trials) by the fourth day of training, the CPLXII KO group did not reach criterion until day 6. Analysis of the number of individual mice reaching criterion on each day revealed that significantly fewer CPLXII KO mice reached criterion on day 4 (15/16 WT compared with 9/15 CPLXII KO, \(\chi^2 = 5.044\)) and day 5 (15/16 WT compared with 9/15 CPLXII KO, \(\chi^2 = 5.044\)).

When the task was reversed, both WT and CPLXII KO mice continued to swim toward the previously reinforced visual cue, the light stimulus. However, WT mice quickly learned to dissociate the original visual cue from reinforcement and swim away from the light stimulus, performing to criterion (four out of five correct choices during a given trial bin) within 25 trials. In contrast, CPLXII KO mice failed to learn the reversal task (Fig. 8C; genotype, \(F_{1,29} = 97.18, P < 0.001\); trial bin \(\times\) genotype, \(F_{6,149} = 9.10, P < 0.001\)). By the end of the testing period, their percentage of correct responses had only reached the level of chance.

**DISCUSSION**

CPLXII KO mice appear outwardly normal, and show no histopathological abnormalities in their brains (5,6). However, we have found that they exhibit progressive deficits in both motor and cognitive behavior that would almost certainly lead to their early demise in a hostile or competitive environment. Although CPLXII KO mice reach maturity and are capable of reproducing and rearing young, they show abnormalities in a number of complex behaviors, including exploration, socialization, motor co-ordination, learning and reversal learning. Our findings suggest that, while CPLXII is not essential for development, it is critical for the acquisition of higher cognitive functions in the adult brain.

We became interested in CPLXII because it is depleted in post mortem HD brains (8). However, without a direct link between the HD mutation and CPLXII, it was difficult to assess the contribution that loss of CPLXII made to the neurological deficits in either mouse or human. Indeed, the reported lack of phenotypic abnormalities in CPLXII KO mice (5) suggested...
that the CPLXII changes in the R6/2 mice were epiphenomena. On the other hand, CPLXII KO mice show impairments in LTP (6,14) similar to those seen in the R6/2 mouse (13). Our previous experience showed that the lack of an overt abnormal phenotype does not mean that a mouse has a full repertoire of normal behaviors. Juvenile R6/2 mice appear 'normal' on home cage observation but display measurable abnormalities in motor and cognitive behavior several weeks before the onset of overt symptoms (16,17). Thus, despite the apparent lack of phenotypic defects, we thought it was worth investigating the behavior of CPLXII KO mice further.

For each of the tests we used, apart from tests of strength, we found deficits in the CPLXII KO mice. In some tasks, the deficits were present from the earliest time of testing. In others, performance was normal in the young adult mouse, but deteriorated with age. For example, from earliest time points, CPLXII KO mice exhibited a lack of exploration that was not due to a change in anxiety levels (22). It is possible that CPLXII KO mice lack the coordination required to explore actively, since they eventually showed a wide-based gait that is consistent with impaired coordination. However, this is unlikely to explain the reduced exploratory behavior since CPLXII KO mice were capable of sustained performance on the rotarod until at least 10 weeks of age. The impairments in the open field task appear to reflect a deficit in the acquisition of normal patterns of adult mouse behaviors. Whereas WT mice became habituated to the open field box and increased their exploratory activities with each successive testing, CPLXII KO mice showed little interest in exploring the environment beyond the floor of the box. This was particularly noticeable with respect to rearing behavior in the center of the field (Fig. 2F). Thus, in the open field task, CPLX II KO mice showed a stable performance that appears impaired only when compared with the changing behavior of the WT mice. We suggest that the abnormal behavior of CPLXII KO mice in the open field reflects a generalized deficit in the acquisition of normal adult mouse behavior.

In contrast to performance in the open field, failure of CPLXII KO mice to perform on the rotarod was not due to relative improvements in the WT performance, since CPLXII KO performance deteriorated not only in comparison to that of WT mice, but also to their own previous performance. Since all behavioral tasks measure a mixture of motor, sensory, affective and cognitive behaviors, we thought it important to check that the deficits we saw were not due to reduced physical strength. In the event, we found no evidence of neuromuscular weakness that would explain our findings. When viewed together our data suggest that the deficits observed in the CPLXII KO mice on the ‘motor’ tasks are not due to failure of muscle strength or vitality, but are likely to reflect deficits of higher brain function.

One concern we had in interpreting our behavioral data was the fact that the mice had a mixed genetic background. However, for a number of reasons we do not think that this is likely to account for the progressive deficits we see. First, we examined the behavior of CPLXII KO and wild-type mice on a number of different tests. At 6–8 weeks of age (young adult mice) we found no significant differences in the behavior between the WT and knockout mice. This contrasts with studies where behavioral differences are seen between different strains of mice, where the differences are apparent from early ages (see for example 23). Second, the CPLXII KO mice served as their own controls in both the rotarod and the MWM testing. Thus the impairments in behavior that we reported in the CPLXII KO mice were significant, not only when compared with the WT mice, but also when compared with their own previous performance.

The most striking deficits we saw were in cognitive tasks, and our data suggest that CPLXII is critically important for some
aspects of both learning and memory. The deficits in the Morris water maze are not due to poor motor performance. Swim speed is a good measure of motor performance in the Morris water maze. Even at 1 year of age the swimming performance of CPLXII KO mice was unimpaired. In fact the oldest CPLXII KO mice swam more quickly than the WT mice. The deficits in reversal learning were particularly intriguing, and they suggest that the learning process in CPLXII KO mice is more rigid than in the WT mice. CPLXII KO mice are able to learn a new task (albeit more slowly than the WT mice). However, having learned it, they have difficulty ‘unlearning’ it in order to learn a new variant of the task. It has been suggested that difficulties in reversal learning are the mouse equivalent of human perseverative behavior (24). Since perseveration is seen in neurological disorders in which CPLXII is depleted (schizophrenia and Huntington’s disease), the CPLXII KO mouse will make a good model in which to study these behaviors further. Interestingly, a deficit in reversal learning in the MWM has been shown recently in a knock-in mouse that expresses α calcium-calmodulin dependent kinase II (CAMKII) that cannot undergo inhibitory phosphorylation (T305D) (25). Although mutations that disrupt CAMKII lead to spatial learning deficits in the MWM (26,27), in the T305D mouse there appears to be a dominant negative effect of CAMKII. Notably, as well as reversal learning deficits, LTP is blocked in T305D mice. Since levels of CAMKII are altered in the R6/2 mouse (28), it would be interesting to determine whether or not levels of CAMKII are also altered in the CPLXII KO mouse.

The MWM is commonly used to test spatial memory, and accurate performance depends on intact hippocampal function (29,30). Therefore, the impairments in spatial learning in the MWM are consistent with a role for CPLXII in the hippocampus. However, lesions in other brain regions including striatum, basal forebrain, cerebellum and cerebral cortex have also been shown to impair MWM performance (for references, see 31). Since CPLXII is highly expressed in the hippocampus, cortex, basal ganglia and cerebellum, and all are regions that are important for learning and memory (for reviews see 32–36), the cognitive deficits in the CPLXII KO mouse are unlikely to be solely deficits of hippocampal origin. Further, while the reversal deficits in the MWM were striking, we also saw a pronounced deficit in reversal of discrimination learning in the two-choice swim-tank. Deficits in reversal of discrimination learning are seen following lesions of the medial prefrontal cortex (37,38) and are thought to be dependent on an intact striato-frontal system (for other references, see 31). It is known that, as well as the hippocampus, intact forebrain structures are essential for cognitive flexibility that is necessary for performance of the reversal learning in the MWM (29,39). It seems likely therefore that loss of CPLXII from all of the interconnected regions in which it is normally expressed contributes to the complex cognitive deficits seen in the CPLXII KO mouse.

Our finding that the deficits in the CPLXII KO mouse are progressive is particularly intriguing. The exploratory deficits in the open field suggest that the WT mice, but not the CPLXII KO mice, are becoming accustomed to the open field. However, an improvement of WT performance against a static performance by the CPLXII KO mice cannot explain the defects we see in performance on the rotarod or the MWM. The progressive deterioration of performance in these tasks suggests that CPLXII is essential not only for developing the full repertoire of normal behavior in the adult mouse, but also for maintaining the function of complex behavioral routines in the adult mouse. Because not all of the tasks we used were conducted longitudinally (see Table 3), it is difficult for us to know precisely when the motor deficits appeared and what relationship they had to the changes in cognitive function (or vice versa). For example, it is not clear whether changes in cognitive function precede motor function or appear in parallel. It would clearly be interesting to investigate this further, given the interdependence of motor and cognitive behaviors.

Although the basic senses of the mice do not appear to be impaired (for example, CPLXII KO mice can find a visible platform as easily as WT mice), we did not test sensory function directly. While hearing and olfactory perception are not essential for performance of tasks such as rotarod and visual cliff, they are critically important for ‘normal’ mouse behavior. In particular, sensory function is central to both social and exploratory behaviors. Since both of these behaviors are impaired in the CPLXII KO mice, sensory function of the CPLXII KO mouse would clearly be worth investigating further.

The deficits seen in the CPLXII KO mice are similar in many respects to those in the R6/2 mouse model of HD in which CPLXII is progressively depleted (12). The similarities extend not only to the behaviors in which deficits are seen, but also to their progressive nature and the order in which the deficits appear. For example, in both CPLXII KO mice (this study) and presymptomatic R6/2 mice (17), deficits in performance of the reversal tasks precede impairments in acquisition learning. Since deficits in reversal tasks are pronounced in early HD (40) when CPLXII is already reduced in the caudate nucleus (8), it would be interesting to determine whether or not experimental strategies aimed at increasing CPLXII levels could reverse the early cognitive deficits in HD mice. Cognitive deficits in HD are currently untreatable and CPLXII would make an interesting therapeutic target.

In summary, our findings show that CPLXII is a key player in the mechanisms underlying complex behaviors in the adult mouse, in particular cognitive processes. It is therefore likely to be important for normal function in the human brain. Further, our data support the idea that depletion of CPLXII contributes significantly to neurological impairment in diseases such as HD. Indeed it seems possible that CPLXII levels may determine the extent to which the patient exhibits cognitive symptoms in the early stages of HD. Depletion of CPLXII may also underlie neurological or psychiatric symptoms in other human diseases where changes in one or both CPLXs have already been reported, such as schizophrenia (9–11), bipolar disorder (41), major depressive illness (11) and temporal lobe epilepsy (42). Finally, we would speculate that depletion of CPLXII could contribute to neurological deficits in behavioral disorders such as autism. The clinical profile of autism includes a mixture of psychiatric and neurological symptoms that include movement abnormalities, disturbances in social interactions and perseveration in the absence of obvious brain pathology (43). The CPLXII KO mouse, with its robust physique and unusual repertoire of social motor and
learning deficits, presents a useful animal model in which to study these behaviors.

**MATERIALS AND METHODS**

**Animals**

Mice were taken from a colony of CPLXII KO mice or CPLXII WT controls established in the Department of Pharmacology, University of Cambridge. The founder mice originated from the Max-Planck Institute for Experimental Medicine, Göttingen, Germany as described by Reim et al. (5). For all experiments, homozygous mutants were used. All mice used in this study were F₁ or F₂, inbred on a mixed genetic background (129Ola/C57Bl/6). Two experimental groups were used. (Details of the testing history of each group, see Table 2.) In both experiments a mix of F₁ and F₂ mice was used. In experiment 1, 78% (WT) and 89% (CPLXII KO) of mice were F₁, 22% (WT) and 11% (CPLXII KO) of mice were F₂. In experiment 2, 16% (WT) and 28% (CPLXII KO) of mice used were F₁, 84% (WT) and 72% (CPLXII KO) of mice were F₂. Mice were housed in hard-bottomed polycarbonate experimental cages in groups of 9–16 mice of the same age, genotype and gender. Lighting was controlled on a 12 h light:12 h dark cycle and all mice were tested during the light phase. The housing facility temperature was maintained at 21–23°C and the relative humidity was also controlled (55 ± 10%). The mice had ad libitum access to water and standard dry laboratory food. In addition, mice were given a supplementary feed each morning of a mash prepared by soaking 100 g dry food in 230 ml of tap water until the pellets were soft and fully expanded. Lowered waterspouts were also provided. This feeding regime improves access to food and water and has been shown to be beneficial to R6/2 mice (44).

Previous studies have shown that CPLXII KO mice brains are morphologically normal (5,6). We examined the brains of CPLXII KO mice histologically using a number of immunocytochemical markers (glial acidic fibrillary protein, ubiquitin, huntingtin, calcium binding protein D28K, syntaxin 1, CPLXI and CPLXII) and have found no obvious structural or morphological abnormalities (data not shown).

Experiments were conducted using two separate groups of animals. The first (group 1) consisted of 18 WT (nine male, nine female) and 18 CPLXII KO (nine male, nine female) mice. The second (group 2) consisted of 32 WT (16 male, 16 female) and 32 CPLXII KO (16 male, 16 female) mice. All mice were weaned at 3 weeks of age and handled daily for 2 weeks thereafter to habituate them to the experimenters. The first behavioral tests (see below) were conducted with mice aged 1 month; the last assessments were made when the mice were 1 year old. At 6 months of age, eight mice from group 2 (four WT and four CPLXII KO) were sacrificed for histological studies (not presented here). Therefore for footprint analysis there were only 28 CPLXII KO mice. One other CPLXII KO mouse from group 2 was killed at 16 weeks. Data from this mouse are included up to this time.

**Body weight.** Body weight was measured at weaning (3 weeks) and then weekly from either 4 or 5 weeks until 24 weeks of age and monthly thereafter. As there was no difference in the body weights or growth patterns of mice from the two groups (data not shown), apart from the genotype effect presented in Figure 1, the data are combined for presentation purposes.

**Modified SHIRPA analysis.** Mice from group 2 were tested using a modified version of the primary screen of the SmithKline Beecham Pharmaceuticals; Harwell, MRC Mouse Genome Center and Mammalian Genetics Unit; Imperial College School of Medicine at St Mary’s; Royal London Hospital; St Bartholomew’s and the Royal London School of Medicine; Phenotype Assessment (SHIRPA) protocol (45,46). The protocol used for collecting these data is detailed in Table 1. Mice were tested at 10, 20 and 30 weeks. Materials for the SHIRPA evaluation consisted of a clean cage with corn-cob bedding and shredded paper nesting material, a wire grid cage lid, and a stopwatch. During the procedure, each mouse was removed from the home cage, evaluated individually in the clean cage, and placed back into the home cage. The testing cage was changed between groups to avoid the possibility of olfactory cues affecting behavior.

The data were quantified using a binary scoring system (Table 2). A ‘normal’ behavior received a score of 0. ‘Abnormal’ behavior received a score of 1. This permits a global abnormality score to be determined for each mouse, with a higher overall score corresponding to a greater degree of abnormality. All tests used in the modified SHIRPA protocol were scored except for positional passivity. We did not include this test in the binary score because the scoring of this task was not easily classified as normal/abnormal. Some individual components of the modified SHIRPA were also analyzed separately.

**Behavioral testing.** Mice were trained on a battery of behavioral tests that gave measures for a number of different parameters. These comprised the open field test (locomotor activity, anxiety, neuromuscular strength and exploration), swim tank (swimming, motivation and coordination), footprints (gait and locomotion), rotarod (coordination, balance and neuromuscular strength), hangwire (grip strength), cage escape test (coordination, motivation and neuromuscular strength), visual cliff avoidance test (visual discrimination and learning), Morris water maze (spatial and reversal learning and memory) and two-choice swim tank task (simple discrimination and reversal learning).

**Hangwire.** Individual mice were placed on a wire cage lid and the lid was gently moved back and forth so as to enable the mouse to grip the wire. The lid was then turned upside down, ~6 inches (a height that mice can easily fall and land on their feet without injury) above the surface of the bedding material. Latency to fall onto the bedding was recorded, with a 60 s cut-off time. This test was conducted at 10, 16, 20, 24 and 30 weeks.

**Swim tank.** To monitor swimming speed and efficiency, mice were trained to swim from one end of a water-filled glass tank to a visible escape platform at the opposite end (16,47). The glass tank (90 x 30 x 16 cm) was filled to a depth of 20 cm with water maintained at a temperature of 23°C. The escape platform was made from black Perspex (6 cm square and 20.5 cm high) with the top surface protruding 0.5 cm above...
the water level. A vertical line on the side of the glass marked a horizontal distance 60 cm from the platform. This served as the start line for recording swimming speed. During the 3 day training period, each mouse was given three trials (maximum 120 s each) in which it was placed in the water at one end of the tank and allowed to swim to the escape platform at the opposite end. On the fourth day, mice were given two trials. The time to swim the 60 cm distance and mount the platform, and the mean number of attempts taken to mount the platform were recorded. In addition, it was noted whether mice had good direction (that is, swam directly to the platform or circled back) and good swim style (fluid limb movements and an absence of floating). Mice from group 2 were tested in the swim tank at 14 weeks.

Open field testing. Mice were evaluated in an open field as described by Carter et al. (48). Individual mice were placed in an open-topped plywood box 60 × 60 cm square × 30 cm high, with a white floor marked with black grid lines (divided into 25 × 12 × 12 cm squares). The box was positioned on the floor in the center of the experimental room. During each testing session, individual mice were placed in the central square of the open field and observed for a 10 min period. Parameters measured within the 10 min period and subsequently analyzed included: (1) latency to reach peripheral squares; (2) total number of central squares entered (defined as three or more paws moving into a central square); (3) total number of peripheral squares entered (defined as three or more paws moving into a peripheral square); (4) total number of squares entered; (5) number of rears against the wall (standing up on hind legs using the wall for support); (6) number of rears in the periphery without the wall (standing up on hind legs); (7) number of rears in the center; (8) number of complete grooming cycles; (9) number of fecal boli; and (10) number of urinations.

Grooming cycle. The grooming cycle was defined as a sequence of behaviors that consisted of sitting without support and then (1) face-washing with the forepaws, (2) licking of the

### Table 1. Modified SHIRPA protocol

<table>
<thead>
<tr>
<th>Test</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hair morphology</td>
<td>Normal</td>
<td>Curled</td>
<td>Other</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tail morphology</td>
<td>Normal</td>
<td>Kink</td>
<td>Curl</td>
<td>Other</td>
<td></td>
</tr>
<tr>
<td>Piloerection</td>
<td>None</td>
<td>Coat stands on end</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Face</td>
<td>None</td>
<td>Coat stands on end</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shoulders</td>
<td>None</td>
<td>Coat stands on end</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Back</td>
<td>None</td>
<td>Coat stands on end</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hindlimbs</td>
<td>None</td>
<td>Coat stands on end</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whiskers</td>
<td>Fully present</td>
<td>Medium</td>
<td>None</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Domen face</td>
<td>Absent</td>
<td>Mild</td>
<td>Severe</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lordokyphosis</td>
<td>Absent</td>
<td>Mild</td>
<td>Severe</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Respiration rate</td>
<td>Gasp, irregular</td>
<td>Slow, shallow</td>
<td>Normal</td>
<td>Hyperventilation</td>
<td></td>
</tr>
<tr>
<td>Palpebral closure</td>
<td>Eyes wide open</td>
<td>Eyes half closed</td>
<td></td>
<td>Eyes closed</td>
<td></td>
</tr>
<tr>
<td>Eye fur</td>
<td>Normal</td>
<td>Slightly pale</td>
<td>Very pale</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gait</td>
<td>Normal</td>
<td>Fluid but abnormal</td>
<td>Limited movement</td>
<td>Incapacity</td>
<td></td>
</tr>
<tr>
<td>Pelvic elevation</td>
<td>Markedly flattened</td>
<td>Barely touches</td>
<td>Normal (3 mm elevation)</td>
<td>Elevated (more than 3 mm elevation)</td>
<td></td>
</tr>
<tr>
<td>Tail elevation</td>
<td>Dragging</td>
<td>Horizontally extended</td>
<td></td>
<td>Everted/Straub tail</td>
<td></td>
</tr>
<tr>
<td>Positional passivity</td>
<td>Struggles when held by tail</td>
<td>Struggles when held by neck (finger grip, not scrubbed)</td>
<td></td>
<td>Struggles when supine (on back)</td>
<td>No struggle</td>
</tr>
<tr>
<td>Forelimb clasping</td>
<td>Absent</td>
<td>Present</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hindlimb clasping</td>
<td>Absent</td>
<td>Present</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aggression</td>
<td>None</td>
<td>Provoked biting or attack</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vocalization</td>
<td>None</td>
<td>Provoked during handling</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tremor</td>
<td>None</td>
<td>Mild</td>
<td>Marked</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial activity</td>
<td>Alert, active</td>
<td>Quiet</td>
<td>Asleep</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Righting reflex</td>
<td>No impairment</td>
<td>Lands on side</td>
<td>Lands on back</td>
<td>Fails to right when placed on back</td>
<td></td>
</tr>
</tbody>
</table>

### Table 2. SHIRPA binary scoring system

<table>
<thead>
<tr>
<th>Test</th>
<th>Score = 0</th>
<th>Score = 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hair morphology</td>
<td>Normal</td>
<td>Abnormal</td>
</tr>
<tr>
<td>Tail morphology</td>
<td>Normal</td>
<td>Abnormal</td>
</tr>
<tr>
<td>Piloerection</td>
<td>None</td>
<td>Coat stands on end</td>
</tr>
<tr>
<td>Whiskers</td>
<td>Medium/none</td>
<td>Fully present</td>
</tr>
<tr>
<td>Domen face</td>
<td>Absent</td>
<td>Present</td>
</tr>
<tr>
<td>Lordokyphosis</td>
<td>Absent</td>
<td>Present</td>
</tr>
<tr>
<td>Respiration rate</td>
<td>Normal</td>
<td>Abnormal</td>
</tr>
<tr>
<td>Palpebral closure</td>
<td>Eyes wide open</td>
<td>Eyes closed/half closed</td>
</tr>
<tr>
<td>Eye fur</td>
<td>Normal</td>
<td>Pale</td>
</tr>
<tr>
<td>Gait</td>
<td>Normal</td>
<td>Abnormal</td>
</tr>
<tr>
<td>Pelvic elevation</td>
<td>Normal (3 mm elevation)</td>
<td>Flattened/elevated</td>
</tr>
<tr>
<td>Tail elevation</td>
<td>Horizontal</td>
<td>Dragging/elevated</td>
</tr>
<tr>
<td>Forelimb clasping</td>
<td>Absent</td>
<td>Present</td>
</tr>
<tr>
<td>Hindlimb clasping</td>
<td>Absent</td>
<td>Present</td>
</tr>
<tr>
<td>Aggression</td>
<td>None</td>
<td>Provoked biting or attack</td>
</tr>
<tr>
<td>Vocalization</td>
<td>None</td>
<td>Provoked during handling</td>
</tr>
<tr>
<td>Tremor</td>
<td>Absent</td>
<td>Present</td>
</tr>
<tr>
<td>Initial activity</td>
<td>Alert, active</td>
<td>Quiet/Asleep</td>
</tr>
<tr>
<td>Righting reflex</td>
<td>No impairment</td>
<td>Fails to right</td>
</tr>
</tbody>
</table>
Chest and/or the forequarters and (3) scratching of the hind-limbs. A grooming cycle consisted of these three actions in this order and each individual cycle was counted. Each of the individual parameters was also scored individually (e.g. face washing by itself or scratching by itself) but only complete grooming cycles were included in the results.

Mice from group 1 were tested in the open field at 4 weeks and then biweekly until 16 weeks.

Footprint analysis. The footprint test was used to compare the gait of CPLXII KO mice with that of WT control mice from group 2 at 8 months of age. Briefly, to obtain footprints, the hind- and forefeet of the mice were coated with purple and orange non-toxic paints, respectively. The floor of the open field box was covered with white paper. Each mouse was placed in a corner of the open field and allowed to walk across the paper, leaving a track of footprints. If a mouse didn’t walk forwards, it was considered to have failed the task. The following parameters were measured: stride length (the mean distance of forward movement between each stride) and base width (the mean distance between left and right footprints). These were determined by measuring the perpendicular distance of a given step to a line connecting its opposite preceding and proceeding steps. For each step parameter, three values were measured from each run, excluding footprints made at the beginning and end of the run where the animal was initiating and finishing movement respectively (for further details see 16). The mean value of each set of three values was used for analysis.

Visual cliff avoidance. Visual cliff avoidance was tested in an open-topped Perspex box (60 × 60 cm square × 15 cm high; Fig. 4A). The box was positioned on the edge of a laboratory bench so that half of the base covered the bench (‘bench side’) while the other half was suspended over the edge of the bench 90 cm above the floor (‘cliff side’), creating the appearance of a cliff without an actual drop-off. A laminated checkerboard pattern was placed under the bench side of the box as well as on the floor underneath the box to emphasize the cliff drop-off. The main light source in the experimental room was dimmed, and Anglepoise lamps (60 W) were positioned 50 cm below and 30 cm above the base of the box to illuminate both the bench and cliff sides of the box. Individual mice were placed on a small (10 × 7 × 2 cm) Perspex block in the middle of the base at the edge of the cliff (on the bench side), allowing the mice to survey their surroundings before dismounting the platform. Their activity was recorded for 5 min from time of placement on the platform. Mice from group 2 were tested at 16 weeks. Parameters analyzed included the latency to dismount the start platform, the direction of the first foot off the platform (that is the cliff or bench), the total time spent on the cliff.
side of the apparatus in the first 2 min and overall, and the percentage of mice remaining on the platform for the entire 5 min trial.

**Rotarod.** The rotarod apparatus (Accelerating model, Ugo Basile, Biological Research Apparatus, Varese, Italy) was used to assess motor coordination, strength and balance (26). During the training period, mice received four trials (maximum 60 s each) per day, at 24 rpm for three consecutive days. The latency to fall off the rotarod within this time period was recorded. On the fourth day, mice underwent testing. During testing, each animal received two trials (maximum 60 s each) at 8 increasing speeds (5, 8, 15, 20, 24, 31, 33 and 44 rpm). The mean latency to fall off the rotarod (for the two trials at each speed level) was recorded and used in the subsequent analysis. Mice from group 1 mice were tested at weekly between 5 and 10 weeks, then again at 24, 28 and 40 weeks. Mice from group 2 mice were tested once only, at 24 weeks of age.

**Cage escape.** Mice were removed from home cages and placed into a clean cage base with fresh corncob bedding. The cage lid was removed and the cage was quickly placed within the open field apparatus. The number of times each mouse climbed up on the sides of the cage was recorded over a period of 10 min. Each time the mice climbed to the top of the box they were replaced on the floor of the cage. Mice from group 1 were tested at 16 weeks and again at 36 weeks, while mice from group 2 were tested at 16 weeks and again at 24 weeks.

**Morris water maze.** Spatial learning was assessed in a MWM modified for use in mice (17). A circular water tank, constructed from white polypropylene (diameter, 120 cm; height, 50 cm) was filled to a depth of 30 cm with water (23°C) rendered opaque by the addition of a small amount of non-toxic white paint. Four positions around the edge of the tank were arbitrarily designated as North (N), South (S), East (E), and West (W), providing four alternative start positions and dividing the tank into four quadrants: NE, SE, SW and NW. A circular clear Perspex escape platform (diameter, 10 cm) was submerged 0.5 cm below the water surface and placed at the midpoint of one of the four quadrants. Four dark blue screens (4 × 120 × 120 cm) surrounded the apparatus to minimize extra-maze cues. Visible cues were placed on three of the screens (black and white checkerboard pattern, four black stars on a white background, and four black circles on a white background). Mice from group 1 were tested in the MWM at 8–9 weeks of age and again at 1 year. Mice were trained for four trials per day with an inter-trial interval of ~10 min. Mice were tested daily over 16 days. On days 1–7, they were trained using a hidden platform. Mice were permitted up to 60 s to locate the escape platform, and their escape latency, path-lengths, swim speed and thigmotaxis (wall hugging) activity were recorded. The HVS tracker system was used to track mice (HVS Image 2020, Hampton, UK). Mice that failed to locate the platform within the time limit were assigned an escape latency of 60 s, placed on the platform by hand and allowed to remain on the platform for 15 s before being removed and returned to the home cage. The escape platform was placed in the midpoint of the SW quadrant (at 8–9 weeks) and in the SE quadrant (at 1 year), and the start position was pseudorandomized across trials.

A probe trial was performed on day 8 to determine if the trained mice showed a preference for the quadrant containing the platform. For this, the escape platform was removed from the tank, and the swimming path of each mouse was recorded over 60 s while it searched for the missing platform. The number of crossings of the platform position was also recorded.

Following the probe trial, reversal training was conducted for a further 7 days. During reversal training, the escape platform was moved to the midpoint of the opposite quadrant (NE for testing at 8–9 weeks and NW for testing at 1 year). The mice were trained to swim to this new position for four trials per day. A second probe trial was conducted on day 16.

Mice were given 3 days’ rest and then tested on a new platform position (SW), for 1 day only, using a visible platform. The platform was made visible by the attachment of a high-contrast black pole. As for the invisible platform, mice were given four trials and the start position was pseudorandomized across trials.

**Two-choice swim tank.** Acquisition of a simple visual discrimination task was assessed using a variation of the two-choice swim tank as described previously (17). The swim tank was filled with water (23°C) and surrounded by 45 cm-high black boards to obscure surrounding spatial cues in the experimental room. Two vertical lines on the sides of the swim tank marked a horizontal distance 36 cm from either end of the tank and provided an 18 cm start area in the middle. The escape platform was placed in a pseudorandom order at either the left or right end of the swim tank for each trial. At the start of each trial, individual mice were placed in the start area facing one side wall to eliminate directional bias. During acquisition training, a 60 W Anglepoise lamp was positioned over the escape platform. During reversal learning, the light was positioned over the end of the swim tank opposite to the platform. The main light source in the experimental room was dimmed to provide a greater contrast between the lit and unlit ends of the swim tank. Mice were trained to swim toward the light stimulus to reach the escape platform in acquisition training, and away from the light stimulus in reversal training.

During acquisition training, mice were given 10–20 trials per day (with an inter-trial interval of ~5 min) for 7 days. On each trial, the mouse was considered to have made a correct choice if it swam directly toward the platform. An incorrect choice was recorded if the mouse swam out of the start area in the opposite direction or if the mouse initially ventured in the correct direction but returned across the start area. Analysis was based on the percentage of correct choices of the first 10 trials performed each day. Training trials were given until the mice made 10 correct choices to a maximum of 20 trials in total. The performance criterion was set at 9/10 correct choices on the first 10 trials. After acquisition training, mice were allowed to rest for 3 days before undergoing a single day of reversal training. During reversal training, each mouse was given a total of 30 trials (with an inter-trial interval of ~5 min). Data were analyzed in blocks of five trials, and the mean number of correct choices within each trial block was used for analysis. The performance criterion was set at achieving four correct trials in a five trial block within the 30 trials. However, because
data were collected blind, all mice received 30 trials, irrespective of whether or not they reach criterion before the 30 trials were completed. Group 2 female mice were tested in the two-choice swim tank at 30–31 weeks of age.

### Statistical analysis

Most behavioral data were subjected to ANOVA, with one or two between-subject factors (genotype, gender and age or trial number) and with repeated measures on one or more factors depending on the test used. Sidák’s test was used for multiple post hoc pair-wise comparisons between CPLXI KO mice and WT controls at each relevant age and test level. Within each group, changes in performance over time were evaluated using Dunnett’s test. Proportions were compared using contingency tables and P-values were calculated using the χ² test. An unpaired two-tailed t-test was applied to test the significance of differences between means where factorial ANOVA was not required. Comparisons of WT and CPLXI KO data from the modified SHIRPA test were analyzed using a Mann–Whitney two-tailed test. Details of statistical significance given in the text refer to two-way ANOVA with Sidák’s post hoc test unless otherwise stated. In most tests there were no significant genotype × gender interactions. Consequently, although the data from males and females were separated in all analyses, data have been pooled for clarity of presentation of the results. Where there are sex differences, these are described in the appropriate results section. Statistical analyses were performed using GraphPad Prism (Version 2.0, San Diego, CA, USA) and Genstat (Release 4.1, NAG Ltd, Oxford, UK). A critical value for significance of P < 0.05 was used throughout the study.

### REFERENCES