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Reduced reactivity to novelty, impaired social behavior, and enhanced basal synaptic excitatory activity in perforant path projections to the dentate gyrus in young adult mice deficient in the neural cell adhesion molecule CHL1

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The neural cell adhesion molecule CHL1 is implicated in neural development in the mouse and has been related to psychiatric disorders in humans. Here we report that mice constitutively deficient for CHL1 display reduced reactivity to environmental stimuli and reduced expression of social behaviors, whereas cognitive, motor and olfactory functions are normal. Basal synaptic transmission and plasticity in seven major excitatory connections in the hippocampus were analyzed to test whether dysfunctions in this brain region, which controls complex behaviors, correlate with the behavioral alterations of CHL1 deficient mice. We found that basal synaptic transmission in lateral and medial perforant path projections to the dentate gyrus is elevated in CHL1-deficient mice. Taking in consideration the function of these synapses in processing information from cortical areas, we hypothesize that constitutive ablation of CHL1 leads to reduced capability to react to external stimuli due to dysfunctions in the dentate gyrus.

Introduction

The immunoglobulin superfamily recognition molecule CHL1 (close homolog of L1) is expressed in the nervous system and, as shown in mice, is developmentally regulated in its expression (Hillenbrand et al., 1999; Holm et al., 1996). CHL1 is expressed by neurons in most brain regions and, unlike some other members of the L1 family, is also synthesized by astrocytes and oligodendrocyte precursor cells. *In vitro*, CHL1 promotes neurite outgrowth of hippocampal and cerebellar neurons (Hillenbrand et al., 1999). To study the function of CHL1 *in vivo*, mutant mice constitutively deficient for CHL1 (CHL1−/− mice) have been generated (Montag-Sallaz et al., 2002). In agreement with the idea that CHL1 is important for the development of the nervous system, CHL1−/− mice show alterations in the organization of hippocampal mossy fibers and of olfactory axon projections (Montag-Sallaz et al., 2002), and displacement of pyramidal neurons in layer V of the visual cortex (Demyanenko et al., 2004).

Identification of a patient with non-specific mental retardation and a mutation in the CHL1 gene has recently provided direct evidence for a link between CHL1 locus and mental functions (Frints et al., 2003). Linkage analyses have also indicated that CHL1 is one of the susceptibility factors for schizophrenia (Chen et al., 2005; Lewis et al., 2003; Sakurai et al., 2002). Similar to schizophrenic patients, CHL1−/− mice are impaired in prepulse inhibition (Irintchev et al., 2004), a measure for the ability of the central nervous system to gate the flow of sensory information (van den Buuse et al., 2003).

Since the hippocampal formation plays a pivotal role in sensorimotor processes (Bast and Feldon, 2003) and is of particular interest in the neuropathology of schizophrenia (Harrison and Eastwood, 2001), we have recently tested the hypothesis that CHL1 is important for the early development and function of this brain region by analyzing morphological and electrophysiological properties of the CA1 field of the hippocampus of 3-week-old CHL1−/− mice. We found an increase in inhibitory postsynaptic currents evoked in pyramidal cells by minimal stimulation of perisomatically projecting interneurons, when compared with wild type (CHL1+/+) littermates (Nikonenko et al., 2006). Also, long-term potentiation (LTP) at CA3–CA1 excitatory synapses was reduced under physiological conditions in these juvenile CHL1−/− mice. This abnormality was abolished by application of a $GABA_A$ receptor antagonist, suggesting that enhanced inhibition is the cause

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of LTP impairment. Quantitative ultrastructural and immunohistochemical analyses also revealed aberrations possibly related to the abnormally high inhibition observed in CHL1−/− mice. The length and linear density of active zones in symmetric synapses on pyramidal cell bodies, as well as the number of perisomatic puncta containing inhibitory axonal markers were increased. Density and total number of parvalbumin-positive interneurons was also abnormally high (Nikonenko et al., 2006).

The aim of this study was to test whether the morphological and electrophysiological abnormalities in the CA1 region of 3-week-old CHL1−/− mice have an impact on behavior in early adulthood, an age at which first episodes of psychosis are typically observed in humans (Thompson et al., 2004). We have, therefore, characterized the behavior of 2- to 3-month-old CHL1−/− mice, with particular emphasis on functions known to be altered in schizophrenic patients, such as novelty response (Laurens et al., 2005), working memory (Miyakawa et al., 2003), social behavior (Erbel-Sieler et al., 2004), and olfaction (Moberg et al., 1999). Moreover, we analyzed whether some of the electrophysiological alterations in the CA1 region of juvenile CHL1−/− mice persist in adulthood. Since the dentate gyrus and the hippocampal regions CA1 and CA3 are considered to regulate memory function and detection of novelty, we analyzed synaptic transmission and plasticity in several major excitatory synapses in these hippocampal regions, aiming to correlate synaptic and behavioral features of CHL1−/− mice.

Results

Behavioral analysis

A longitudinal study including tests for exploration and anxiety, motor coordination, cognition, and social behavior was carried out on CHL1−/− male mice and their CHL1+/+ littermates. A batch of CHL1−/− female mice and CHL1+/+ littermates underwent the social preference and olfaction tests.

Open field

In this test, motor activity and exploratory behavior of mice is commonly indicated by their distance moved, mean velocity and rearing on the hind limbs. As the centre of an arena is an anxiogenic stimulus for mice, thigmotaxis (the tendency to stay and move along the wall of the arena) is used to measure anxiety. No differences between the genotypes were detected for distance moved, mean velocity and time in the center of the open field. Mice of both genotypes decreased the distance moved over the 15-min test (Fig. 1A), indicating normal short-term habituation to the new environment. Whereas mean distance to the wall and time in the border region of the open field (two parameters for thigmotaxis) did not differ between the genotypes as calculated for the total 15-min duration of the test, two-way ANOVA for repeated measurements showed a significant effect of the interaction between Genotype and Time interval: Post-hoc analysis revealed that CHL1−/− mice stayed at longer distances from the wall during the second 5-min interval (Fig. 1B) and spent less time in the border during the last 10 min of the task as compared with their CHL1+/+ littermates (Fig. 1C). Moreover, CHL1−/− mice reared less when compared with CHL1+/+ mice as indicated by a general effect of Genotype and by the effect of the interaction between Genotype and Time interval: Post-hoc analysis indicated that CHL1−/− mice reared less than CHL1+/+ mice during the first 2 min of the test (Fig. 1D).

Light/dark avoidance test

Mice tend to avoid illuminated areas where they can be seen by possible predators. In the light/dark avoidance test, the propensity to approach a brightly illuminated area is used to measure anxiety in mice. Mice of both genotypes spent more time in the “red quadrant” as compared with the chance levels of 25% with no difference between genotypes (40±4.9% for CHL1+/+ and 38±3.8% for CHL1−/− mice).

Elevated-plus maze test

The elevated-plus maze test is a classical test to measure anxiety in rodents in which a reduced or enhanced exploration of the open arms of the maze is interpreted as higher or lower anxiety, respectively. No difference was detected between the genotypes for most of the parameters analyzed: mice of both genotypes showed equal amount of locomotion as indicated by frequency of total transitions (16.8±1.6 for CHL1+/+ and 17.0±1.4 for CHL1−/− mice) and closed arms entries (12.7±0.8 for CHL1+/+ and 11.9±1.2 for CHL1−/− mice). Both groups spent equal amounts of time on the open arms and entered into the open arms equally frequently (Fig. 1F and G). CHL1−/− mice reared less as compared with CHL1+/+ mice (Fig. 1E). No differences were found between genotypes for the other parameters analyzed.

New object test

This test was performed to test novelty-seeking and anxiety by measuring the propensity of mice to approach and investigate a new object introduced into their territory. CHL1−/− mice appeared to perceive the new object more slowly than CHL1+/+ mice, as indicated by the higher latency to display the first sniffing event towards the object (Fig. 1H). CHL1−/− mice spent more time inside the object as compared with CHL1+/+ mice (Fig. 1I), although both genotypes spent equal amounts of time at the object (data not shown). No differences were found between genotypes for the other parameters analyzed.

Urine marking

Urine marking is an important behavior that regulates social interactions in mice. Male mice usually mark the territory by leaving small urine drops not only to communicate their possession of the territory, but also to inform female mice of the presence of a reproductive male mouse. CHL1−/− mice showed a drastic reduction of urine marking behavior as compared with CHL1+/+ mice (Fig. 2E). Analysis of the spatial distribution of urine drops indicated that the mice of both genotypes marked the area in the proximity of the females more intensely when compared with the other parts of the open field (data not shown). Mice of both genotypes spent most of the time in the proximity of the females, although CHL1−/− mice needed more time to develop a preference for the females' quadrant as indicated by the fact that they spent less time near the females during the first 5 min of the test as compared with CHL1+/+ mice (Fig. 2F).

Resident/intruder test

The resident/intruder test was performed to test aggressive behavior towards an unfamiliar male mouse, a typical intrasexual competitive behavior expressed by male mice in order to defend their territory and submit conspecific intruders. CHL1−/− mice showed higher latencies to bite the intruder as compared with CHL1+/+ mice (Fig. 2A). Since this difference could be due to the fact that there were two more non-aggressive mice in the CHL1−/−

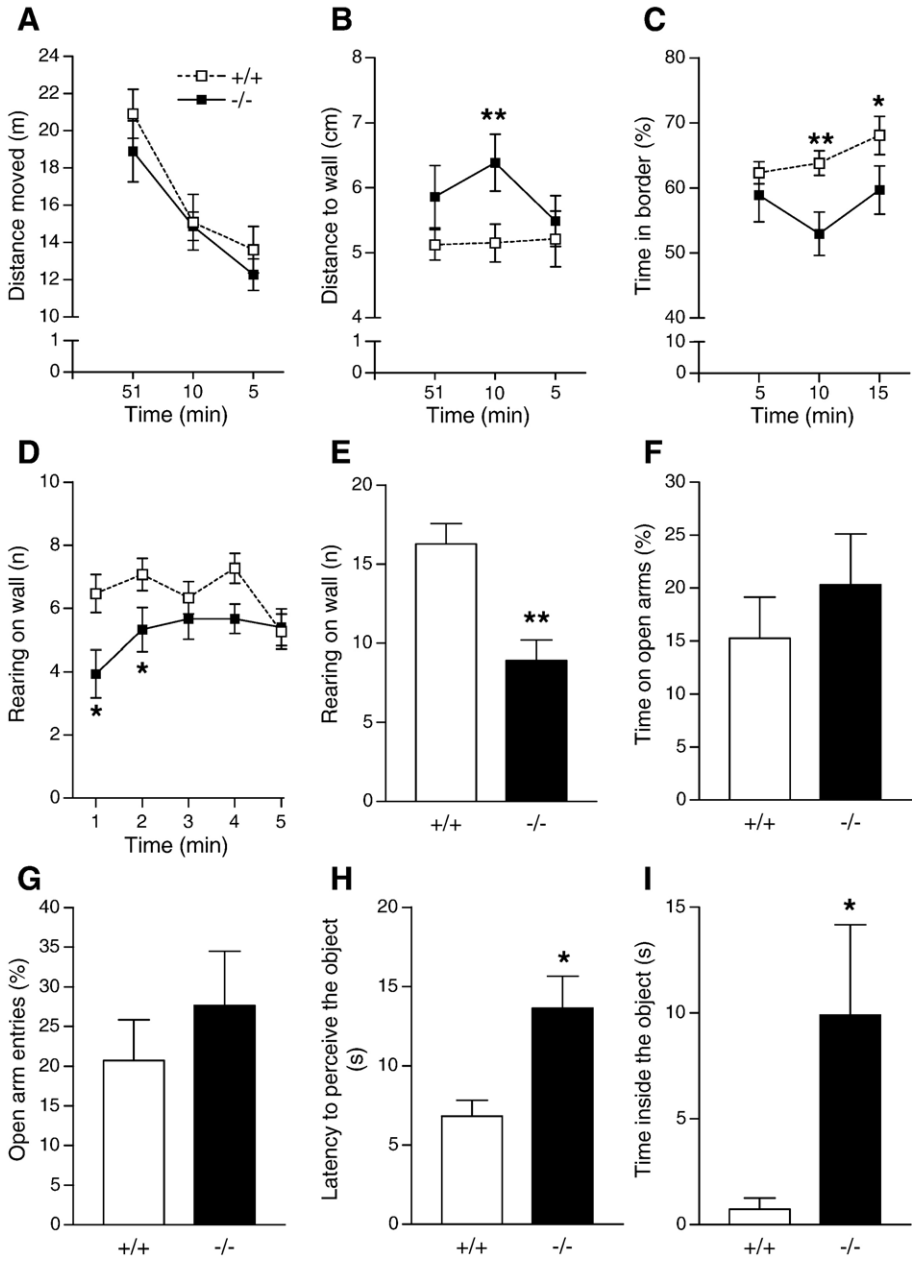

Fig. 1. Reduced novelty-induced behavior of CHL1−/− mice in the open field, elevated-plus maze and new object tests. (A) No difference was detected for distance moved in the open field: both genotypes declined the distance moved over the three 5-min intervals of the test, indicating unaltered locomotion and short-term habituation in CHL1−/− mice. (B–C) CHL1−/− mice showed slightly reduced thigmotaxis as compared with CHL1+/+ mice as indicated by their higher distance to the walls during the second 5-min interval (B) and more time spent in the center of the arena during the last 10 min of the test (C). (D) CHL1−/− mice reared less as compared with CHL1+/+ mice during the first 2 min of the open field test. (E–G) In the elevated-plus maze, CHL1−/− mice reared less than CHL1+/+ mice (E), while their exploration of the open arms was unaltered, as indicated by the time spent in the open arms (F) and the percentage of open arm entries (G). (H–I) In the new object test, although CHL1−/− mice needed more time to perceive the new object (as measured by sniffing directed towards the new object) (H), they spent more time than CHL1+/+ mice inside the object (I). $^{*}p<0.05$, $^{**}p<0.01$ as compared with CHL1+/+ mice (panels B–D: measured with the Neuman–Keuls post-hoc after two-way ANOVA for repeated measurements; panels E, H and I: measured with the Mann–Whitney *U*-test).

as compared with the CHL1+/+ group (non-aggressive mice were given a maximal latency to bite of 600 s), we calculated the latency to bite only for the CHL1+/+ and CHL1−/− mice that had bitten the intruder: Still, CHL1−/− mice needed more time to bite the intruder as compared with CHL1+/+ mice (Fig. 2B). Moreover, CHL1−/− mice bit the intruder less than CHL1+/+ mice (Fig. 2C). CHL1−/− mice also spent less time in social investigation of the intruder as compared with CHL1+/+ mice (Fig. 2D). No differences were found between genotypes for the other parameters analyzed.

Vertical pole and rotarod tests

No differences were found between the genotypes in terms of motor function and coordination as tested in the vertical pole and rotarod tests. In the vertical pole test, all mice were capable to turn

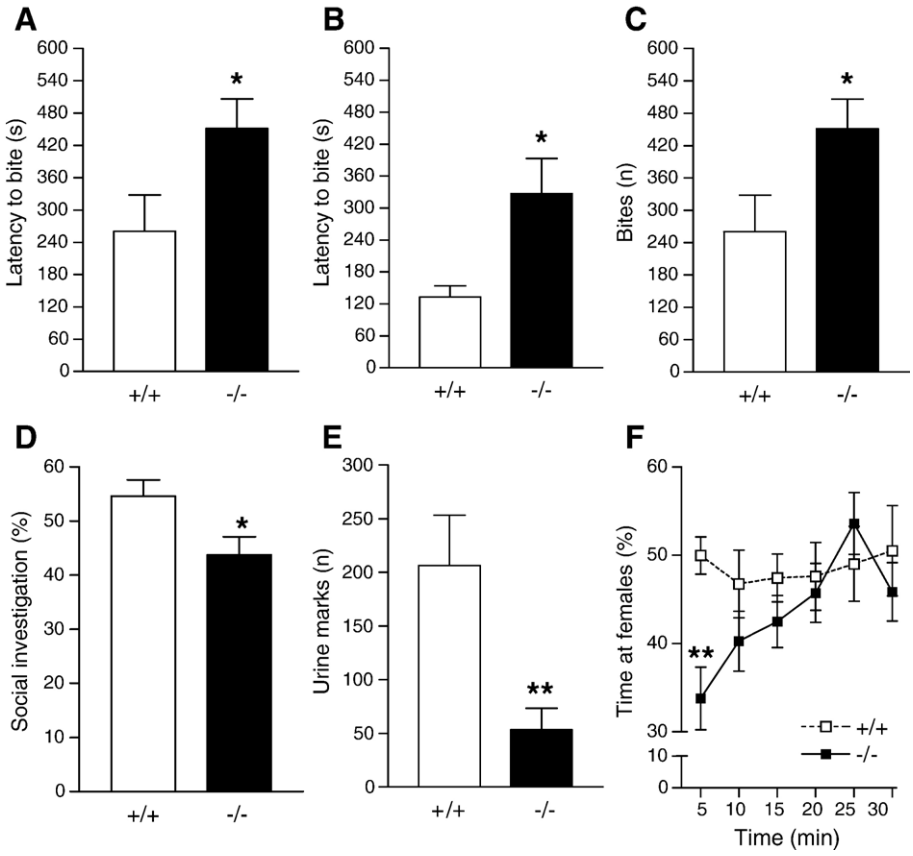

Fig. 2. Reduced aggressive, social, and urine marking behavior in CHL1−/− mice. (A) In the resident–intruder test, CHL1−/− mice showed a higher latency to bite the intruder as compared with CHL1+/+ mice (mice not biting scored a latency of 600 s: 3 CHL1+/+ mice and 5 CHL1−/− mice did not bite the intruder). (B) Also when only mice biting the intruder were considered, CHL1−/− mice showed higher latency to bite as compared with the CHL1+/+ mice. (C–D) CHL1−/− mice directed less bites towards the intruder (C) and spent less time in social investigation towards the intruder (D) as compared with CHL1+/+ mice. (E–F) In the urine marking test, CHL1−/− mice made less marks (E) and spent less time in contact with the female mice during the first 5-min interval of the test (F) as compared with CHL1+/+ mice. $^{*}p<0.05$, $^{**}p<0.01$ as compared with CHL1+/+ mice (panels A–E: measured with the Mann–Whitney *U*-test; panel F: measured with the Neuman–Keuls post-hoc after two-way ANOVA for repeated measurements).

the body by 180° and climb down the pole, a motor performance that requires good coordination in the three dimensions. In the rotarod test, motor function and coordination is measured by the ability of mice to move on a rod that rotates with an accelerating speed. There was no effect of Genotype or of the interaction between Genotype and Trial on latency to fall, indicating that CHL1−/− and CHL1+/+ mice performed equally well in this task (data not shown). Both genotypes increased their performance over trials as indicated by a significant effect of the factor Trial on the latency to fall, suggesting that CHL1+/+ and CHL1−/− mice are capable of cerebellum-dependent motor learning.

Step-through passive avoidance test

The step-through passive avoidance test is a one-trial learning task that is considered to require hippocampal function (Izquierdo and Medina, 1997). Mice were conditioned to passively avoid a dark compartment in which they received a foot-shock as they stepped through during the conditioning trial. When tested for retention 24 h after conditioning, mice of both genotypes significantly increased the latency to step through as compared with the latency shown at conditioning (Fig. 3A). Thus, both CHL1+/+ and CHL1−/− mice learned to avoid to step into the dark compartment and were capable to retrieve this information after a long-term time interval after conditioning.

Spontaneous alternation test

The spontaneous alternation is a classical test for working memory in rodents: rodents tend to visit places they have not explored before. For instance, if a mouse has explored the left arm of a T-maze, it is expected to enter into the right side the next time it is exposed to the maze, i.e. alternation occurs in animals that remember which was the last visited arm. The capacity to store information for longer periods of working memory can be tested by manipulating the duration between trials (inter-trial interval). To test mice under a less and more challenging protocol, mice underwent two sessions with an inter-trial interval of 5 s and two sessions with an inter-trial interval of 30 s, respectively. Since the same results were obtained from the sessions with the same inter-trial interval, data were pooled together for the 5- and 30-s inter-trial interval (Fig. 3B, C). CHL1−/− mice and CHL1+/+ littermates exhibited alternation behavior as shown by the percentage of alternations that was higher than the chance level of 50% for both inter-trial intervals. Mice of both genotypes displayed higher alternations with an inter-trial interval of 5 s as

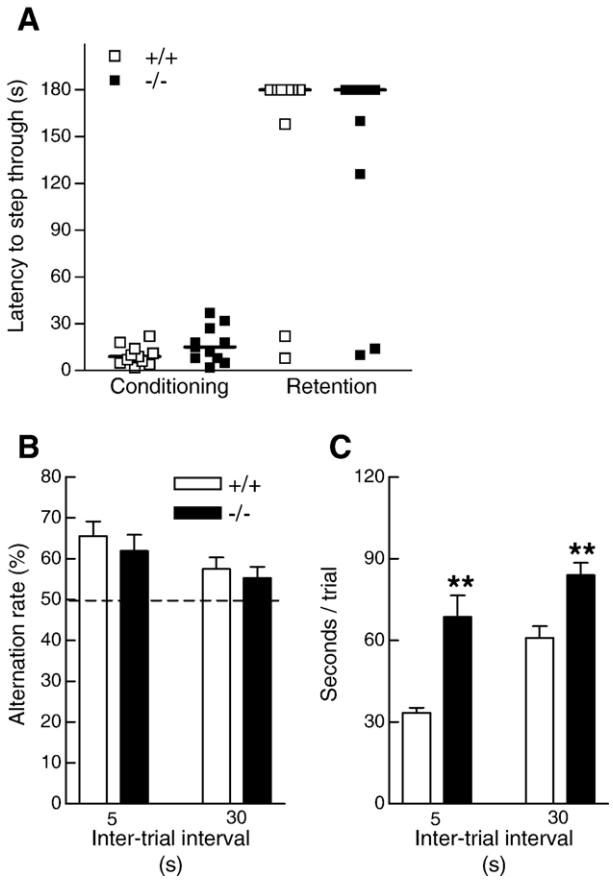

Fig. 3. Normal long-term and working memory in CHL1−/− mice. (A) In the step-through passive avoidance task, both genotypes avoided to step through during the retention test performed 24 h after conditioning, indicating normal one-trial learning and long-term memory. For both genotype groups, values for each individual and median value of the group are indicated. (B) In the spontaneous alternation test, both genotypes showed an alternation behavior with 5 s and 30 s inter-trial intervals, as indicated by an alternation ratio higher than the chance level of 50% (indicated by the dotted line). (C) CHL1−/− mice needed more time to perform a trial in the spontaneous alternation test as compared with CHL1+/+ mice. $^{**}p<0.01$ as compared with CHL1+/+ mice (Mann–Whitney *U*-test).

compared with 30 s, indicating that working memory declines over a longer interval (Fig. 3B). The only genotype-related difference detected was that CHL1−/− mice needed more time than CHL1+/+ littermates to complete one trial (calculated from the moment when the door at the starting arm was opened to the moment the mouse returned into the starting arm after it had entered one of the opposing arms) (Fig. 3C).

Social preference test

Mice tend to investigate more intensively unfamiliar mice as compared with familiar ones. In this test, social preference is tested by measuring the time mice spent investigating familiar versus unfamiliar mice. After the test was started, CHL1−/− and CHL1+/+ female mice first approached the familiar females and then the unfamiliar ones, indicating that both genotypes could distinguish between familiar and unfamiliar individuals. CHL1−/− mice needed more time than CHL1+/+ mice to start exploring the cages with both the unfamiliar and familiar females (Fig. 4A), indicating a delayed reactivity to initiate social investigation. CHL1+/+ mice preferentially investigated the unfamiliar females, while CHL1−/− mice spent equal time investigating the two groups of females (Fig. 4B). Moreover, CHL1+/+ mice spent more time in the compartment of the unfamiliar females than in the compartment of the familiar females, whereas CHL1−/− mice spent equal amount of time in both compartments and less time in the compartment of the unfamiliar females as compared with CHL1+/+ littermates (data not shown).

Test for olfactory function

This test was made to determine the olfactory function of mice by measuring their ability to detect the presence of a food reward by use of olfaction. All mice promptly ate all the pieces of chocolate during the pre-training session, indicating their high motivation to find the reward. As only difference between genotypes, CHL1−/− mice needed more time to complete the two trials on day 1 during which five pieces of chocolate were placed at the entrance of the tubes (data not shown). On days 2 and 3, both CHL1+/+ and CHL1−/− mice learned to enter into the tube containing the chocolate, as indicated by the fact that the number of errors declined over consecutive trials reaching an asymptotic level on the last five trials on day 3 (Fig. 4C). The two-way ANOVA for repeated measurements did not show any effect of Genotype on number of errors, indicating that CHL1+/+ and CHL1−/− mice used olfactory information equally efficiently. There was an effect of the interaction between Genotype and Trial on number of errors and time required to complete the trials: Post-hoc analyses indicated that CHL1−/− mice made more mistakes than CHL1+/+ mice on trials 2 and 3 (Fig. 4C) and needed more time to eat the chocolate in trials 2–4 and 7 as compared with CHL1+/+ mice (Fig. 4D).

Electrophysiological analysis

To search for electrophysiological correlates of abnormal behavior in CHL1−/− mice, we analyzed synaptic transmission and plasticity in several major excitatory synaptic connections in the hippocampal formation of these mutants.

Analysis of perforant path projections to the dentate gyrus

First, we focused on synaptic connections in the dentate gyrus, which are formed by lateral and medial perforant path fibers originating mostly from neurons located in the layer II of the entorhinal cortex. To find lateral perforant path responses, we placed stimulating and recording electrodes in the outer part of the molecular layer in the dentate gyrus (Fig. 5A) and searched for fEPSPs showing facilitation in response to paired-pulse stimulation with 50 ms inter-stimulus interval (Fig. 5B, inset). The level of facilitation was 118.6±2.9% in CHL1+/+ mice, which was not significantly different from 120.4±2.8% measured in CHL1−/− mice. Two-way ANOVA for repeated measurements with subsequent *t*-tests revealed that, except for the lowest amplitude of fEPSP, fEPSPs of similar amplitude were elicited by lower stimulation intensities in CHL1−/− mice compared with CHL1+/+ mice (Fig. 5B), indicating an increase in basal synaptic transmission in CHL1 mutants. In addition, we analyzed relationships between amplitudes of fEPSPs and presynaptic fiber volley, as a measure related to the number of excited axons. Due to rather high variability in presynaptic volleys no difference between

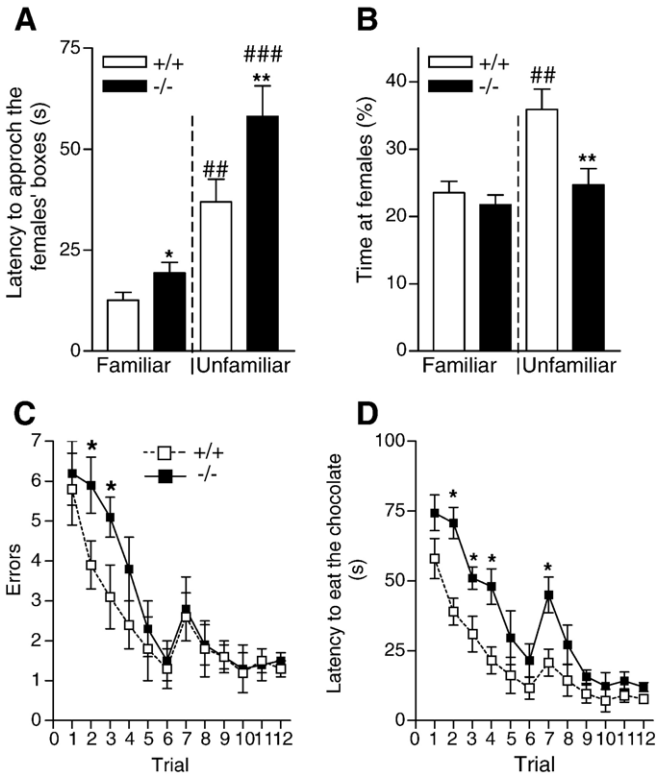

Fig. 4. Altered social preference and normal olfactory function of CHL1−/− mice. (A) Both CHL1+/+ and CHL1−/− female mice first approached the familiar female mice *versus* the unfamiliar mice; CHL1−/− mice showed a higher latency to approach the familiar and unfamiliar boxes as compared with CHL1+/+ mice. (B) CHL1+/+ but not CHL1−/− mice preferentially investigated the unfamiliar mice. (C) In the test for olfactory function, both genotypes found a 0.1 g piece of chocolate by olfaction. CHL1−/− mice made more errors than CHL1+/+ mice in trials 2 and 3. (D) CHL1−/− mice needed more time to find and eat the chocolate in trials 2–4 and 7 as compared with CHL1+/+ mice. In panels C and D, trials 1–6 and 7–12 were performed on 2 consecutive days. $^{*}p<0.05$, $^{**}p<0.01$ as compared with CHL1+/+ mice (Neuman–Keuls post-hoc after two-way ANOVA for repeated measurements); $^{\#}p<0.05$, $^{\#\#}p<0.001$, $^{\#\#\#}p<0.001$ as compared with familiar mice within the same genotype (Neuman–Keuls post-hoc after 2-way ANOVA for repeated measurements).

genotypes was detected. To induce long-term potentiation (LTP) in perforant pathway synapses, five trains of high-frequency stimulation (HFS) were delivered to the slices. There was no difference for short-term potentiation (STP) and LTP between two genotypes (Fig. 5C). The levels of STP were 132.1±8.3% in CHL1+/+ and 131.3±4.5% in CHL1−/− mice. The levels of LTP were 122.0±2.8% in CHL1+/+ mice and 119.9±3.3% in CHL1−/− animals.

To find medial perforant path responses, we placed stimulating and recording electrodes in the inner part of the molecular layer in the dentate gyrus (Fig. 5D) and searched for fEPSPs showing depression in response to paired-pulse stimulation with 50 ms inter-stimulus interval (Fig. 5E, inset). Paired-pulse depression was 81.2±0.8% in wild type animals and 80.2±1.4% in CHL1−/− mice. Thus, no difference was detected. However, it should be noted that since we analyzed inputs with paired-pulse modulation below or above 100% for medial and lateral perforant path projections, respectively, we introduced a bias in selection of inputs. Therefore, some of the abnormal inputs showing, for instance, depression instead of facilitation might be excluded from consideration. However, the analysis performed was meaningful, since it would not preclude the detection of differences between wild-type and CHL1-deficient mice in the distributions of paired-pulse modulation values, if these differences lay in the range below or above 100% for medial and lateral perforant path projections, respectively.

Two-way ANOVA for repeated measurements detected a highly significant difference between stimulus–response curves in CHL1+/+ and CHL1−/− mice (Fig. 5E): the p value for the effect of genotype on stimulus intensity was 0.007. In agreement with these data, there was a tendency for CHL1−/− mice to exhibit elevated amplitudes of fEPSPs as a function of fiber volley amplitude ($p=0.093$). Delivery of five trains of HFS to slices elicited similar degrees of STP and LTP in the two genotypes (Fig. 5F). The levels of STP were 141.2±11.7% in CHL1+/+ mice and 144.0±4.6% in CHL1−/− mice. Values of LTP were not different between CHL1+/+ (147.3±11.5%) and in CHL1−/− mice (138.1±7.2%).

Thus, synaptic plasticity is normal, whereas basal synaptic transmission appeared to be elevated in both lateral and medial perforant path projections to the dentate gyrus of CHL1−/− mice.

Analysis of perforant path projections to the CA3 and CA1 fields

Neurons in the layer II of the entorhinal cortex not only send perforant path fibers to the dentate gyrus, but also project to the CA3 field that provides monosynaptic activation of the distal dendrites of CA3 pyramidal cells (Fig. 6A). Analysis of perforant path projections to the CA3 field showed that there was no statistically significant difference in the stimulus–response curves between CHL1−/− and CHL1+/+ mice (Fig. 6B). Also no difference was detected for paired-pulse facilitation with 10, 20, 50, 100 and 200 ms inter-stimulus intervals (Fig. 6C). Two trains of HFS delivered to the slices evoked STP of 193.1±5.2% in

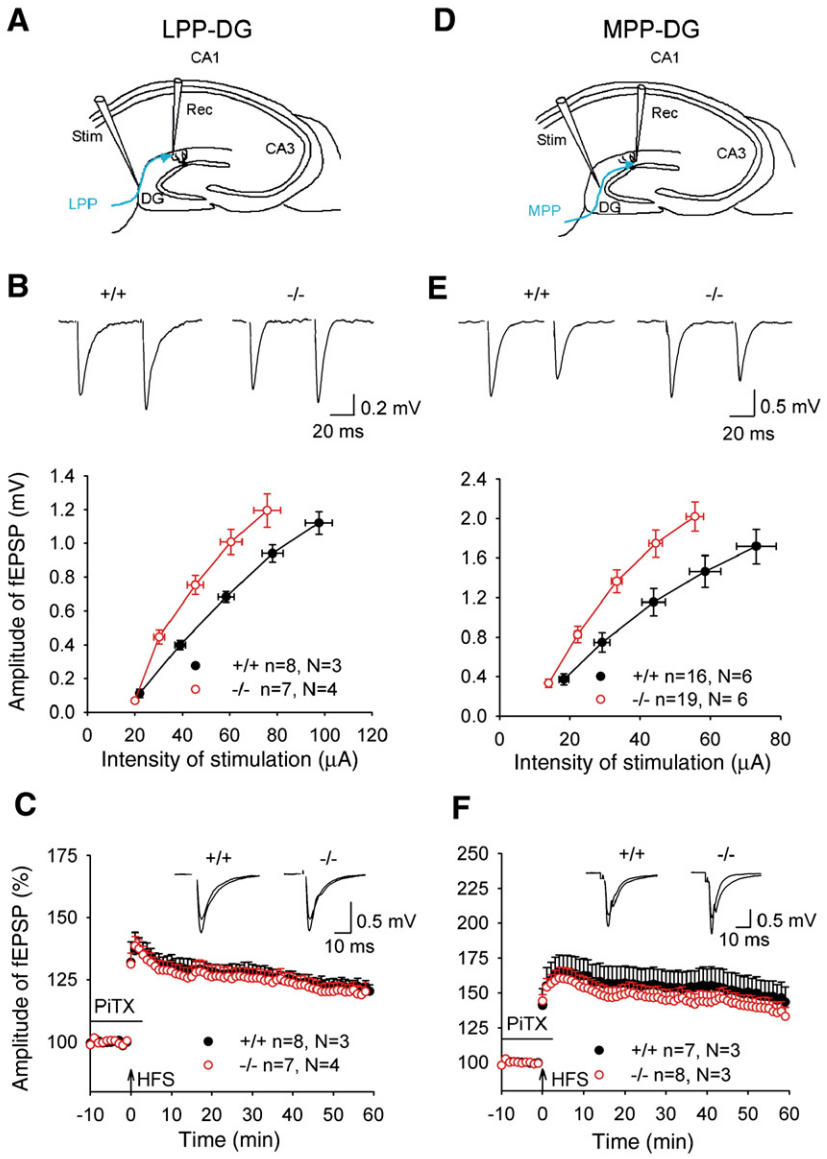

Fig. 5. Synaptic physiology of perforant path connections in the dentate gyrus of CHL1−/− and CHL1+/+ mice. (A and D) Location of stimulating (Stim) and recording (Rec) electrodes used to elicit and record fESPSs in the lateral (A) and medial (D) perforant path (LPP and MPP, respectively) connections. (B and E) Input–output curves for amplitudes of fEPSPs evoked by stimulation of lateral (B) and medial (E) perforant path projections to the dentate gyrus at different stimulation strengths. CHL1−/− mice exhibit elevated levels of basal synaptic transmission. Insets show paired-pulse modulation of fEPSPs. (C and F) STP and LTP were induced by five trains of high-frequency stimulation (HFS) applied to lateral (C) and medial (F) perforant path projections in the presence of $GABA_A$ receptor antagonist picrotoxin (PiTX). No difference in synaptic plasticity between genotypes was observed. Insets show fEPSPs recorded before and 50–60 min after HFS for both genotypes. *N* represents the number of tested mice, *n* is the number of tested slices.

CHL1+/+ and 183.5±7.5% in CHL1−/− mice. Values for LTP were not different between CHL1+/+ (114.2±2.5%) and CHL1−/− mice (112.8±3.1%) (Fig. 6D).

CA1 pyramidal neurons also receive synapses from perforant path projections (Fig. 6E). These fibers originate from the layer III of the entorhinal cortex. We detected no statistically significant difference in the stimulus–response curves between two genotypes (Fig. 6F). Analysis of paired-pulse facilitation with 10, 20, 50, 100 and 200 ms inter-stimulus intervals also did not reveal any abnormalities in CHL1−/− versus CHL1+/+ mice (Fig. 6G). To induce LTP in perforant path synapses in CA1, two trains of HFS were delivered to the slices. This stimulation induced STP of 186.2±4.5% in CHL1+/+ and 189.6±4.1% in CHL1−/− mice. The levels of LTP were 111.7±3.3% in CHL1+/+ and 110.9±3.1% in CHL1−/− mice. Thus, no statistically significant difference was observed for STP and LTP between the genotypes (Fig. 6H).

Analysis of CA3 projections to the CA3 and CA1 fields

CA3 pyramidal cells have associational/commissural axons projecting to CA3 pyramidal neurons (Fig. 7A). Two-way ANOVA for repeated measurements did not reveal any differences between CHL1+/+ and CHL1−/− mice with regard to stimulus–response curves (Fig. 7B) and paired-pulse facilitation (Fig. 7C). Two HFS induced synaptic plasticity in CA3–CA3 synapses in both

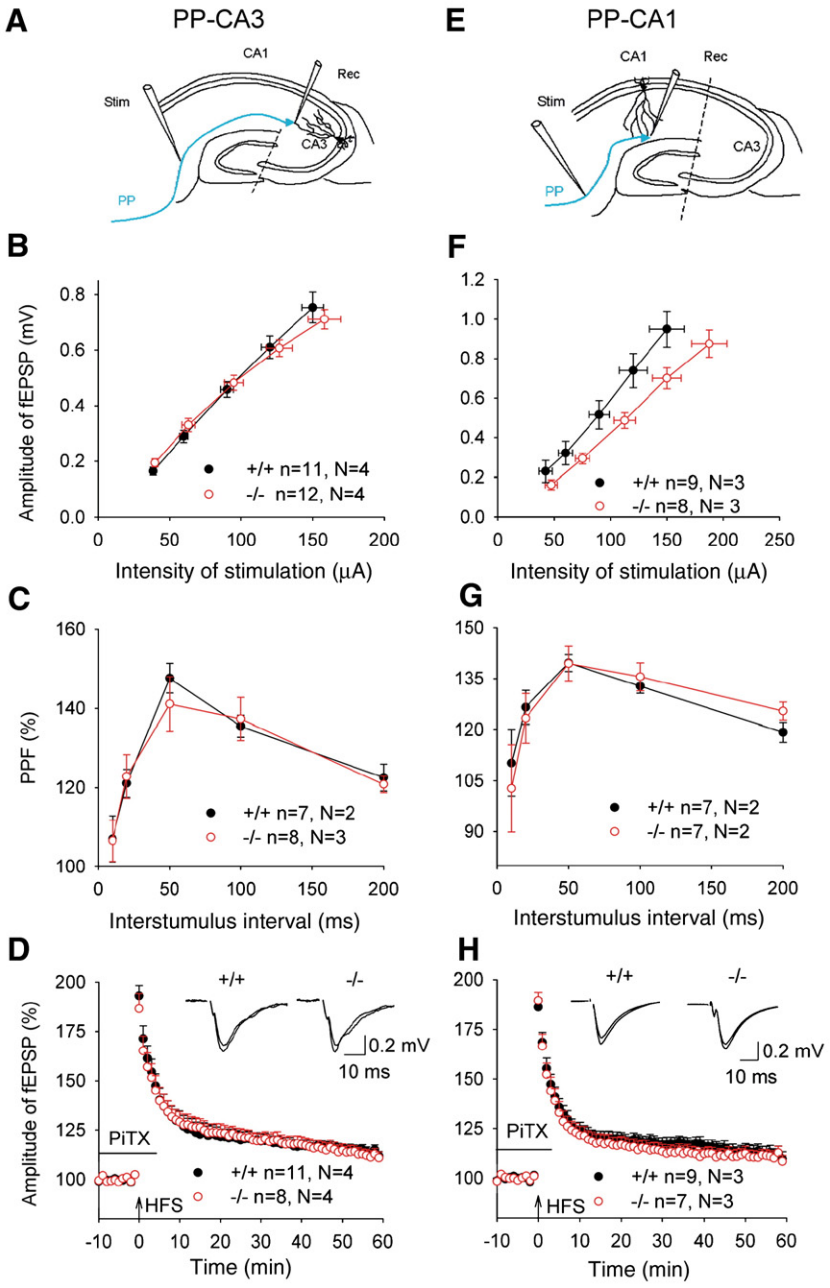

Fig. 6. Synaptic physiology of perforant path (PP) connections in the CA3 and CA1 regions of CHL1−/− and CHL1+/+ mice. (A and E) Location of stimulating (Stim) and recording (Rec) electrodes used to elicit and record fESPSs in the CA3 (A) and CA1 (D) regions. (B and F) Input–output curves for amplitudes of fEPSPs evoked by stimulation of perforant path and recorded in the stratum lacunosum moleculare of CA3 (B) and CA1 (F) regions. (C and G) Paired-pulse facilitation (PPF) with 10, 20, 50, 100 and 200 ms inter-stimulus intervals in the CA3 (C) and CA1 (G) regions. (D and H) STP and LTP were induced by two trains of HFS in the CA3 (D) and CA1 (H) regions in the presence of $GABA_A$ receptor antagonist picrotoxin (PiTX). No difference between genotypes was detected. Insets show fEPSPs recorded before and 50–60 min after HFS for both genotypes. *N* represents the number of tested mice, *n* is the number of tested slices.

genotypes. The levels of STP were 173.7±6.9% in CHL1+/+ mice and 177.5±7.5% in CHL1−/− mice. The levels of LTP were 130.5±3.6% in CHL1+/+ and 123.5±3.4% in CHL1−/− mice (Fig. 7D). Statistically significant differences were found neither for STP nor for LTP.

Information from the CA3 to CA1 field is transmitted via Schaffer collaterals to the ipsilateral hippocampus (Fig. 7E) and via commissural projections to the contralateral hippocampus. No differences in stimulus–response curves (Fig. 7F) or in paired-pulse facilitation (Fig. 7G) were detected in these synaptic connections. Two trains of high frequency stimulation induced STP and LTP in CA3–CA1 synapses in mice of both genotypes. STP in CHL1+/+ mice was 178.5±5.0% and 181.8±10.0% in CHL1−/− mice. Levels of LTP were 115.6±3.6% and 112.3±2.4% in CHL1+/+ and CHL1−/− mice, respectively (Fig. 7H). Thus, the impairment of STP and LTP previously observed in juvenile (3-week-old)

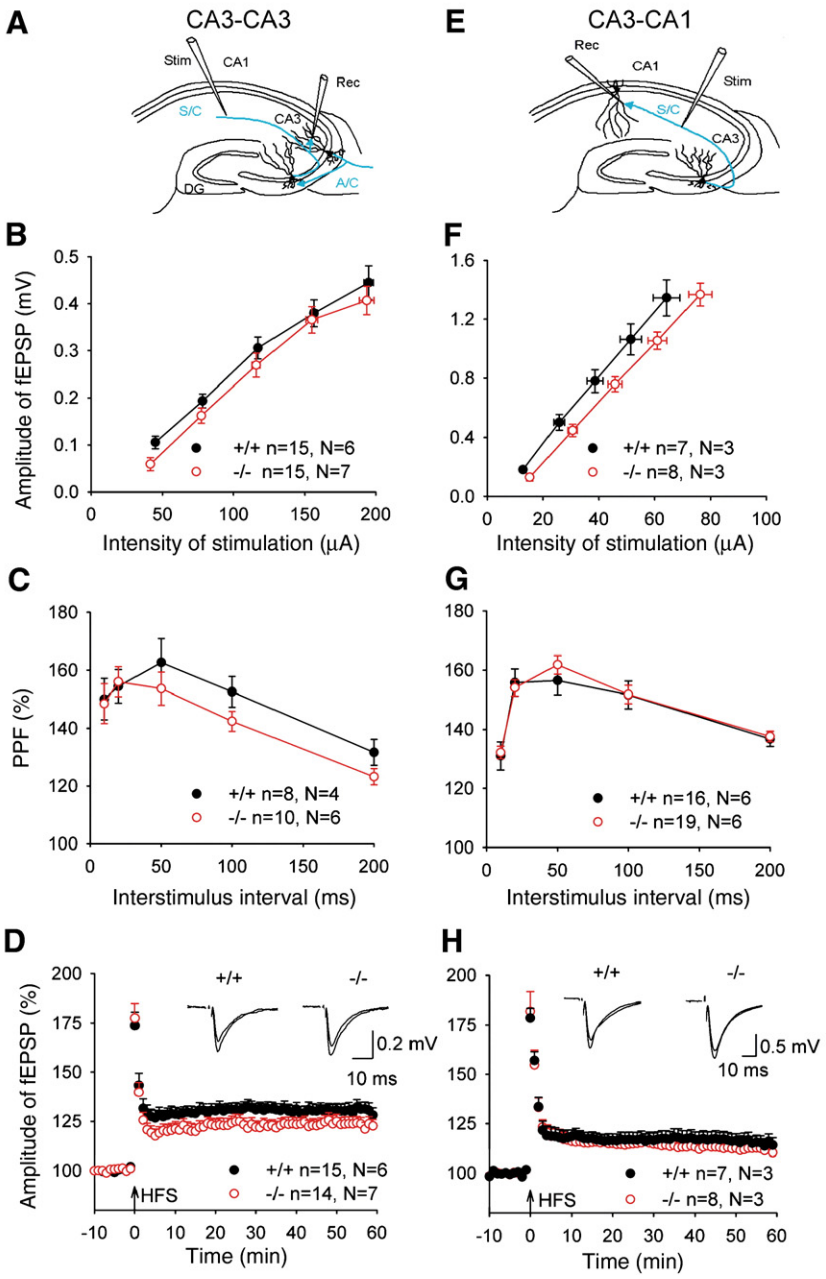

Fig. 7. Synaptic physiology of CA3 connections in the CA3 and CA1 regions of CHL1−/− and CHL1+/+ mice. (A and E) Location of stimulating (Stim) and recording (Rec) electrodes used to elicit and record fESPSs in the CA3 (A) and CA1 (D) regions. (B and F) Input–output curves for amplitudes of fEPSPs evoked by stimulation of axons of CA3 pyramidal cells and recorded in the stratum radiatum of CA3 (B) and CA1 (F) fields. (C and G) Paired-pulse facilitation (PPF) with 10, 20, 50, 100 and 200 ms inter-stimulus intervals in the CA3 (C) and CA1 (G) regions. (D and H) STP and LTP were induced by two trains of HFS in the CA3 (D) and CA1 (H) regions. No difference between genotypes was detected. Upper panel shows fEPSPs recorded before and 50–60 min after HFS for both genotypes. *N* represents the number of tested mice, *n* is the number of tested slices.

CHL1−/− mice (Nikonenko et al., 2006) was not present in young adult (2-month-old) mice.

Analysis of mossy fiber projections to the CA3 field

Mossy fibers transmit signals from the dentate gyrus to the CA3 field (Fig. 8A). Analysis of the physiological characteristics of mossy fiber projections to the CA3 field in CHL1+/+ and CHL1−/− mice revealed that there was no difference between the genotypes in terms of frequency-dependent facilitation in response to 0.33 Hz stimulation and a blockade of responses by an agonist of metabotropic glutamate receptors DCG IV (Fig. 8B, C), which are two characteristic features of mossy fiber fEPSPs. Another important feature is that mossy fiber LTP is NMDA receptor independent, unlike LTP in the aforementioned connections. Two trains of HFS delivered to the slices in the presence of the NMDA receptor antagonist APV induced similar levels of post-tetanic potentiation and LTP in slices from CHL1−/− and CHL1+/+ mice. The profiles of LTP were similar to those previously reported for

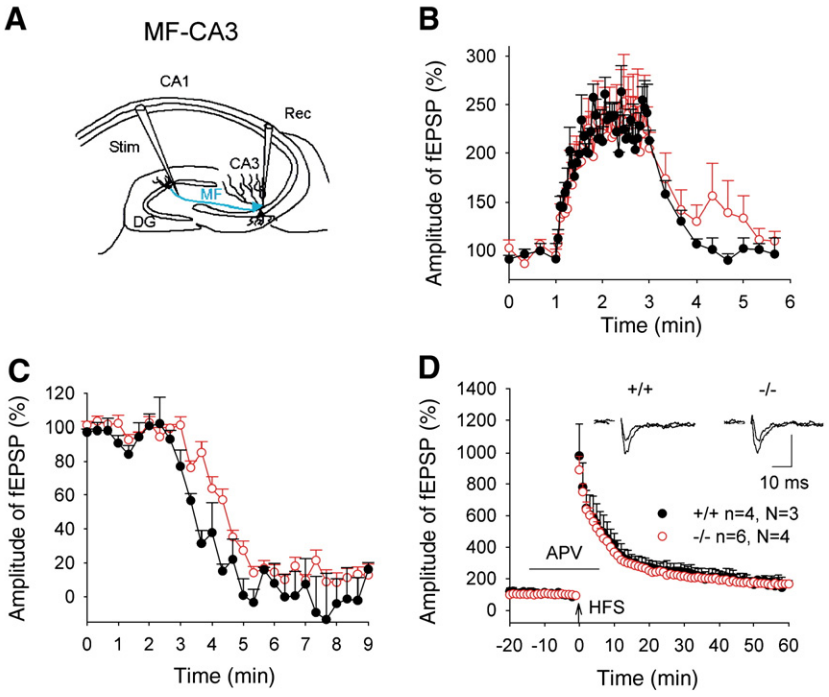

Fig. 8. Synaptic physiology of mossy fiber (MF) connections in the CA3 region of CHL1−/− and CHL+/+ mice. (A) Location of stimulating (Stim) and recording (Rec) electrodes used to elicit and record mossy fiber fESPSs in the CA3 region. (B) Frequency-dependent facilitation of fEPSPs recorded in the stratum lucidum in response to mossy fiber stimulation at 0.33 Hz. (C) Inhibition of responses with the group II mGluR agonist DCG IV. (D) Post-tetanic potentiation and LTP induced by two trains of HFS in the presence of the NMDA receptor antagonist APV. No difference between genotypes was detected. Insets show fEPSPs recorded before and 50–60 min after HFS for both genotypes. *N* represents the number of tested mice, *n* is the number of tested slices.

wild type mice (Evers et al., 2002). Post-tetanic potentiation was 977±198% in CHL1+/+ and 887±87% in CHL1−/− mice. The values for LTP were 168.7±38.1% in CHL1+/+ and 166.9±4.6% in CHL1−/− mice (Fig. 8C). Thus, despite alterations of hippocampal mossy fiber organization in CHL1−/− mice (Montag-Sallaz et al., 2002), all investigated synaptic properties of mossy fiber–CA3 connections appeared to be normal.

Discussion

We report that basal synaptic transmission is enhanced in the dentate gyrus of CHL1−/− mice, whereas LTP is normal in the dentate gyrus as well as in the CA3 and CA1 regions of the hippocampus. At the behavioral level, CHL1−/− mice show reduced social behavior and response to environmental stimuli, whereas anxiety- and cognitive-related behaviors appear to be normal.

Unaltered anxiety-related behaviors in CHL1−/− mice

Anxiety-related parameters did not differ between CHL1−/− and CHL1+/+ littermates as measured in the open field, light/dark avoidance, elevated-plus maze, and new object tests, indicating unaltered anxiety under CHL1 deficiency, although the slightly reduced thigmotaxis of CHL1−/− mice in the open field test could be interpreted as reduced anxiety as previously suggested (Montag-Sallaz et al., 2002). These results thus partly confirm and extend those previously described in three independent studies using the open field, elevated-plus maze and light/dark avoidance tests that showed no major alterations in anxiety levels of CHL1−/− mice (Montag-Sallaz et al., 2002; Pratte et al., 2003; Frints et al., 2003). As the only discrepancy to our study, Montag-Sallaz et al. (2002) reported that CHL1−/− mice were less anxious in the elevated-plus maze, although CHL1−/− mice differed from CHL1+/+ littermates only for the parameter open arm entries divided by closed arm entries. However, in agreement with our results, in the latter study no genotype-related differences were found for the most commonly accepted parameters for evaluation of anxiety, namely time spent in the open arms and number of entries into the open arms normalized by total transitions.

Reduced reactivity to environmental stimuli and impaired social behavior in CHL1−/− mice

It is noteworthy that rearing behavior was drastically reduced in CHL1−/− mice as compared with CHL1+/+ mice in the open field, elevated-plus maze and new cage tests. Since rearing is a typical novelty-induced response expressed within few minutes after exposure to a new environment, the reduced rearing of CHL1−/− mice suggests that CHL1−/− mice respond less to environmental stimuli. This alteration could also explain the reduced reactivity in the tests for spontaneous alternation and for olfactory function, and the delayed response to social stimuli in the urine marking, resident–intruder, and social preference tests. Rearing has been correlated with structural variations in the mossy fiber terminal field of the hippocampus in mice (Crusio et al., 1989a; van Daal et al., 1991) and rats (Prior et al., 1997; for review, see Lever et al., 2006). In particular, mice selected for high frequency of rearing behavior in an open field showed larger infrapyramidal mossy fiber terminal fields as compared with mice selected for low expression

of rearing behavior (Crusio et al., 1989b). Reduced rearing behavior may therefore be a consequence of the abnormally targeted mossy fiber projections observed in CHL1−/− mice (Montag-Sallaz et al., 2002).

Another important observation of this study was that social behavior of CHL1−/− male and female mice was restrained. Since in CHL1−/− mice olfactory sensory axons are misguided (Montag-Sallaz et al., 2002), reduced and retarded response to social and non-social stimulation could be due to sensory deficits. However, we tend to exclude this possibility since mice of both genotypes distinguished between the smell of familiar and unfamiliar mice and detected an invisible food reward by olfaction. As mentioned, reduced expression of social behavior measured in this study could in part be explained by the delayed initiation of appropriate behavioral responses after presentation of a conspecific animal. In this sense, the reduced social behavior of CHL1−/− mice could arise from their deficit in attention and sensory gating rather than from a specific impairment of social behavior.

The observation that CHL1−/− mice react more slowly to environmental and social stimuli strongly suggests impaired attention or inability to detect salient information from the environment in these mice. It is noteworthy to mention in this context that reduced sustained attention is characteristic of schizophrenic patients (Sponheim et al., 2006; Laurent et al., 1999; Lenzenweger et al., 1991). Interestingly, CHL1 heterozygous and homozygous deficient mice show reduced prepulse inhibition of acoustic startle response, indicating an impaired ability to gate the flow of sensory information, another typical behavioral marker for schizophrenia (Irintchev et al., 2004). Therefore, ablation of CHL1 in mice leads to behavioral alterations that are similar to some of those in schizophrenic patients. These observations suggest that CHL1−/− mice may represent a model for some traits observed in schizophrenia in humans (Frints et al., 2003; Sakurai et al., 2002).

It is conceivable that reduced attention and/or detection of novelty by CHL1−/− mice arise as a consequence of deficits in the circuitry underlying attention and sensory gating involving brain regions such as the sensory and prefrontal cortices, the superior colliculus, pons, nucleus accumbens, amygdala and, last but not least, the hippocampus (Swerdlow et al., 2001). The fact that deep layer pyramidal neurons in the visual and somatosensory cortices of CHL1−/− mice are displaced to lower laminar positions and develop aberrant dendrites (Demyanenko et al., 2004) suggests that CHL1 regulates area-specific neuronal function in posterior neocortical areas.

Enhanced basal synaptic transmission in perforant path projections to the dentate gyrus of CHL1−/− mice

In agreement with previous studies, we did not find any cognitive alterations in CHL1−/− mice versus their CHL1+/+ littermates in the step-through passive avoidance and spontaneous alternation tests. This observation goes along with normal synaptic transmission and plasticity in acute hippocampal slices of 2- to 3-month-old CHL1−/− mice: STP and LTP at many synapses of the hippocampal circuitry (i.e., perforant path–dentate gyrus, perforant path–CA1, perforant path–CA3, mossy fiber–CA3, CA3–CA1 and the autoassociative CA3–CA3 synapses) were normal. The absence of any impairment in synaptic plasticity in hippocampal slices of young adult CHL1−/− mice is surprising since CHL1 is strongly expressed in the hippocampus during development and in adulthood and, in particular, in neurons in the CA1 field and the dentate gyrus (Hillenbrand et al., 1999; Nikonenko et al., 2006). It is interesting in this respect that also mice constitutively deficient for L1, another member of the L1 family of neuronal recognition molecules, show normal hippocampal synaptic transmission and plasticity in the CA1 region as measured *in vitro* and *in vivo* (Bliss et al., 2000).

The observations of normal physiological responses are unexpected since perisomatic inhibition of CA1 pyramidal neurons is enhanced resulting in reduced LTP in 3-week-old CHL1−/− mice (Nikonenko et al., 2006). The normal levels of LTP in 2- to 3-month-old mice observed in this study suggest that the abnormally high perisomatic inhibition is reduced during postnatal development as a result of compensatory mechanisms or retardation of the accelerated maturation of the GABAergic system in CHL1−/− mice. Because the recordings in slices from 2- to 3-month-old mice are very challenging, we could not directly measure perisomatic inhibition at this age. However, our interpretation is strongly supported by morphological data showing that the abnormally high numbers of parvalbumin-positive interneurons present in the CA1 field and in the whole hippocampus of 1-month-old CHL1 deficient mice are reduced to normal levels by the age of 2 months (B. Thilo, A. Irintchev and M. Schachner, unpublished observations).

The only detectable physiological alteration in CHL1−/− mice was that of altered stimulus–response relationship in the dentate gyrus. The intensity of stimulation required to elicit synaptic responses in CHL1−/− slices was smaller compared with CHL1+/+ preparations, indicating enhanced basal excitatory synaptic transmission in the dentate gyrus of CHL1−/− mice. The findings of normal LTP and enhanced basal transmission are not inconsistent, since it has been reported that changes in basal activity do not necessarily lead to abnormalities in STP or LTP (Fitzjohn et al., 2001). Similarly, in the CA1 field of conditionally deficient L1 mice LTP was normal despite elevated basal excitatory synaptic transmission (Law et al., 2003).

Concluding remarks

The most remarkable behavioral alteration that we observed in CHL1−/− mice was a delayed responsiveness to environmental stimuli, suggesting that CHL1−/− mice are impaired in extracting relevant information from the environment. In this context, it is interesting that the dentate gyrus plays an important role in processing sensory information conveyed from the cortex (Kesner et al., 2004). The bulk of afferents to the dentate gyrus arise from layer II of the enthorinal cortex in rats and monkeys (Steward and Scoville, 1976; Witter and Amaral, 1991) and it has been hypothesized that these projection play an important role in novelty detection (Lorincz and Buzsaki, 2000). In freely behaving rats, dentate gyrus interneurons largely increase their firing rates in a novel environment (Nitz and McNaughton, 2004), and the induction and maintenance of LTP in the dentate gyrus are enhanced after exposure to a new environment (Davis et al., 2004). Conversely, rats with a lesioned dentate gyrus are unable to recognize new objects introduced into the environment (Lee et al., 2005). It has been suggested that the perforant path regulates the response of the dentate granule cells to sensory input from the neocortex by decreasing the synaptic drive when granule cell activity is high and increasing this synaptic drive when granule cell activity is low (Hampson and Deadwyler, 1992). Thus, we

speculate that enhanced basal excitatory synaptic transmission in the CHL1−/− dentate gyrus reflects a dysfunction in the regulation of granule cell activity altering the input–output function for sensory stimulation in such a way that signals from novel stimuli will not be much above the background level, leading to retardation and attenuation of novelty responses in CHL1−/− mice.

Thus, our extensive physiological analysis indicates that, while synaptic plasticity in major excitatory connections is normal in the adult hippocampus, basal synaptic transmission in perforant path projections to the dentate gyrus is enhanced in CHL1−/− mice, providing a possible functional correlate to their retarded behavioral response to environmental and social stimuli. These observations support previous results indicating impaired sensory gating in CHL1−/− mice, and provide further indication that dysfunction in the human CHL1 could be responsible for the expression of some symptoms typical of psychiatric disorders such as schizophrenia.

Experimental methods

Behavioral analysis

Husbandry and animals

CHL1 wild-type (+/+) mice and CHL1-deficient (−/−) 10-week-old littermates from heterozygous breeding pairs (mixed C57BL/6J×129Ola genetic background, 4 backcrosses into C57BL/6J) were transferred from the breeding facility into a *vivarium* with an inverted 12:12 h light/dark cycle (light off at 7:00 a.m.) and maintained under standard housing conditions (21±1 °C, 40–50% humidity, food and water *ad libitum*). All tests were performed during the dark cycle of the animals in a room next to the *vivarium* that was illuminated with dim red light. Tests started and ended at least 3 h after light offset and 3 h before light onset, respectively. The experimental material was cleaned with soap, water, and ethanol (70%) before and after each contact with an animal. To avoid a "litter effect", no more than two animals per genotype were used from the same litter. Eleven CHL1+/+ male mice and 11 CHL1−/− littermates underwent a longitudinal study starting two weeks after they had been accustomed to the animal facility according to the temporal order: open field, elevated-plus maze, new object, urine marking, resident-intruder, spontaneous alternation, step-through passive avoidance, vertical pole, and rotarod tests. Mice were kept single-housed starting from the new object test. Tests were separated by at least a 2-day break (a break of 10 days was applied after the step-through passive avoidance test). Ten CHL1+/+ female mice and 10 CHL1−/− female littermates were tested in the social preference and olfaction tests.

Open field test

The open field test was performed as described (Law et al., 2003) in a box (50×50 cm and 40 cm high) illuminated with white light (50 lx). Mice were started from one corner of the box and tested for 15 min. Tracks were produced and analyzed with the software EthoVision (Noldus, Wageningen, The Netherlands). Behavior during the first 5 min was scored by a trained observer using the software The Observer (Noldus). The following parameters were analyzed: stretch attend posture (the mouse elongates the body forward and then retracts to the original position without stepping forward with the hind limbs), rearing on wall (the mouse stands on the hind limbs and touches the wall with at least one fore paw), rearing off wall (as rearing on wall but with the fore paws not contacting the wall), immobility, and self-grooming.

Light/dark avoidance test

This test was performed in the box described for the open field test. In contrast to the classical light/dark test, the open field had no compartments, and a vertical projector differentially illuminated the floor of the open field: one quadrant (25×25 cm) was illuminated with dim red light (20 lx) and served as dark section named "red quadrant", whereas the other three quadrants of the open field were illuminated with bright white light (600 lx) named "lit quadrants". Mice were started in one of the lit quadrants. The percentage of time spent in the red quadrant and the total distance moved were analyzed for 10 min with the software EthoVision.

Elevated-plus maze test

The maze had the shape of a plus with four 30-cm-long and 5-cm-wide arms, connected by a squared center (5×5 cm). Two opposing arms were bordered by 15 cm high walls (closed arms), whereas the other two arms (open arms) were bordered by a 2-mm rim. The maze was elevated 75 cm from the floor and an infrared camera allowed video-recording under total darkness. The mouse was placed into the center facing one open arm and left on the maze for 5 min. The following parameters were analyzed with The Observer: entries into the open and closed arms (calculated when all the four paws were on an arm), total transitions (the sum of entries into the open and closed arms), entries into the edges of the open arms (calculated when the mouse reaches with its snout the edge of an open arm), latency to enter into the open arms, latency to reach the edge of an open arm, stretch attend posture towards the open arm, rearing, self-grooming, head dips from the "protected" area (head movements over the side of an open arm with the snout pointing downwards while the mouse is still in the center or closed arm), head dips from the "unprotected" area (head dips are done as the mouse is on the open arms), and self-grooming.

New object test

The new object test was performed as described (Brandewiede et al., 2005; Morellini and Schachner, 2006). Mice were placed into a cage (38×22×15 cm) with fresh bedding and food and water *ad libitum*. After 24 h a new object was introduced into the cage. The new object consisted of a plastic water bottle (7×7 cm and 10 cm high) for rodents with the bottom cut off and with an entrance of 3×4 cm on one side. It was placed into the cage with the entrance facing the center of the cage. Behavior was video-recorded for 5 min after the new object had been introduced into the cage. The following parameters were analyzed using The Observer: self-grooming, rearing, digging, climbing (hanging from the top of the cage), eating/drinking, stretch attend posture towards the object, sniff at object (nose in direct contact with the object), rear at object, and time inside the object (entering the object with at least the head).

Urine marking test

Three C57BL/6J female mice were placed in one corner of the open field already described. This corner was separated from the rest of the open field by Plexiglas walls (18×18 cm and 30 cm high) with drilled holes (diameter of 0.8 cm) which allowed visual and olfactory perception but limited body contact between the females and the male. The floor of the arena was covered with Whatman filter paper #4. Mice were placed into the open field where they could freely move for 40 min. Urine spots were counted under UV light. Distance moved, mean velocity and time spent in the quadrant with the female mice were scored with EthoVision.

Resident/intruder test

The resident/intruder test was performed 3 days after the urine marking test and 6 days after being single housed in the cage for the new object test. The test started with an unfamiliar C57BL/6J age- and body weight-matched male mouse (intruder) being placed into the home cage of the experimental male mouse (resident). The behavior of the resident mouse was recorded for 10 min. Latency to initiate, frequency and duration of the following parameters were scored with the software The Observer: social investigation, allo-grooming, chasing, attacking, biting, tail rattling, mounting, rearing, digging and self-grooming.

Spontaneous alternation test

The spontaneous alternation test was performed as already described (Morellini and Schachner, 2006). The maze consisted of three arms of the same size (34×5 cm and 30 cm high): two opposite arms and one central arm, made of transparent Plexiglas and connected such as to make a T. Two

sliding doors controlled the access to the two opposite arms. A sliding door was placed at the edge of the central arm and could be closed in order to confine the mouse at the dead end of this arm. Mice were tested over 4 days, 1 session per day and 14 trials per session. Each session was started by placing the mouse at the dead end of the central arm with the door closed. After 5 s the door was opened and the mouse was allowed to freely enter into one of the two opposite arms. Once the mouse had entered into one arm, access to the opposite one was occluded by the sliding door. As the mouse returned to the dead end of the central arm, it was confined there for 5 s on days 1 and 2, and 30 s on days 3 and 4 before starting the next trial by opening the door. The sessions on days 3 and 4, with an inter-trial interval of 30 s, were designed to challenge working memory abilities more than the sessions on days 1 and 2, when a shorter inter-trail interval of 5 s was used. Data were analyzed as percentage of alternations over all trials.

Step-through passive avoidance test

A two-compartment box equipped with a grid-floor was used. The box was made of white plastic with a sliding door (5 × 5 cm) connecting the two compartments. One smaller compartment (13 × 21 cm and 30 cm high) was illuminated (50 lx) while the other (25 × 21 cm and 30 cm high) remained dark (0.5 lx). On the first day mice were placed into the illuminated compartment. After 1 min the sliding door was opened. After the mouse had encountered the open door for the first time, the latency to enter into the dark compartment was measured. When the mouse had entered the dark compartment with 4 paws, the door was closed and a foot shock (1 s, 0.25 mA) was delivered. Mice were then immediately returned to their home cages. Retention was tested 24 h later by repeating the whole procedure, with the exception that the mice did not receive any foot shock: mice were given 5 min to enter the dark compartment after which the test was interrupted.

Vertical pole test

In the pole test, motor coordination was monitored while mice were climbing down a pole. Mice were placed head upward at the top end of a rough-surfaced vertical wooden rod (60 cm long, 7 mm in diameter). To test motor learning, mice were scored three times with an inter-trial interval of 30 s during which they were placed into their home cage. The time needed to reach the floor with all four paws and the manner by which mice descended from the top of the pole were analyzed as described (Freitag et al., 2003).

Rotarod test

Mice had to walk on a turning, corrugated rod (3.2 cm in diameter) (Acceler Rotarod for mice Jones and Roberts, TSE Systems, Bad Homburg, Germany). Mice underwent 5 trials with an inter-trial interval of 45 min. Trials 1 and 2 were performed at low (4 rpm) constant speed for a maximum duration of 3 min. Trials 3–5 were performed with the accelerating rod, starting with 4 rpm up to 40 rpm within 4 min, with a maximum duration of 5 min. On the following day, a sixth trial on the accelerating rod was carried out. The performance was evaluated by scoring the latency to fall.

Social preference test

In this test, social recognition and social preference for familiar versus unfamiliar conspecific animals was tested by giving the experimental female mouse the choice to stay in proximity of familiar or unfamiliar female mice. Starting from postnatal day 21, mice were kept in groups composed of one CHL1+/+ or one CHL1−/− female mouse with three age-matched C57BL/6J female mice. These three C57BL/6J females were then used during the experiment as familiar females, whereas three age-matched C57BL/6 female mice that had not been in contact with the experimental mouse were used as unfamiliar female mice. The apparatus consisted of two compartments (50 × 25 cm and 40 cm high) divided by a 40-cm-high wall with a circular starting box (diameter of 15 cm, 30 cm high) in the middle. The starting box had two sliding doors to regulate the access to the two compartments. The apparatus was illuminated by dim white light (10 lx). One corner of both compartments was surrounded by a Plexiglas wall, to generate a chamber (18 × 18 cm and 30 cm high) in which either three familiar or unfamiliar females were confined. The Plexiglas walls had holes (diameter of 0.8 cm) at their bottom to allow olfactory exploration between the three C57BL/6J females in the chamber and the experimental female freely moving in the two compartments. To avoid any bias due to the location or non-obvious characteristics of the compartment, the position of familiar and unfamiliar female mice was counterbalanced between the chambers in the two compartments for both genotypes. The experimental CHL1+/+ or CHL1−/− female mouse was placed into the starting box with the doors closed: after 10 s the trial started by lifting the doors so that the experimental mouse had free access to both compartments for 15 min. Distance moved, mean velocity, time spent in each compartment, and time spent investigating the two chambers (measured when the nose contacted the walls of the chambers) were analyzed with the software EthoVision and The Observer.

Test for olfactory function

The maze consisted of an open field (75 × 50 cm and 50 cm high). On one of the 50 cm walls of the maze were placed five tubes (30 cm long and 4 cm in diameter). Each tube was open at one side and closed at its opposite side. The tubes were placed on the floor perpendicular to the wall with the closed side touching the wall and the open side (entrance) facing the center of the maze. Distance between adjacent tubes was 4 cm. A trial started as the mouse was placed inside the open field with the nose facing the wall at the opposite side of the tubes. Each trial ended when the mouse had eaten a piece of chocolate (0.1 g) placed inside one of the tubes. The inter-trial interval was of 30 s during which the mouse was placed back into its home cage. Mice were familiarized to the chocolate 5 days before starting the pre-training phase by placing five pieces of chocolate inside the home cage. On day 1, mice underwent a familiarization session of 4 trials during which they learned to find the chocolate in the tubes: in the first two trials, the chocolate was placed directly in the proximity of the starting position. Then, mice underwent two trials in which one piece of chocolate was placed at the entrance of each tube. The test for olfaction was performed on days 2 and 3 in one session of 6 trials: on each trial, the chocolate was randomly placed inside one of the tubes at its closed extremity. All trials were performed under darkness: thus, the use of olfactory stimuli was the only one that the mouse was allowed to use to detect whether a tube contained the chocolate or not. As error was considered when a mouse entered with four paws a tube not containing the chocolate. Numbers of errors and latency to find the chocolate were calculated for each trial.

Analysis of behavioral parameters

All tests except the grip and rotarod tests were video-recorded. Tracks representing the position of the mice were created and analyzed with the software EthoVision as described (Freitag et al., 2003). Analysis of behavior with the software The Observer was performed by a trained experimenter blind to the genotype. The experimenter trained himself until he repeatedly scored at least 80% of consistency between two analyses performed at different times on the same mouse, as calculated with the Reliability Test provided by the software The Observer (having 2 s as maximal time discrepancy between two evaluations).

Electrophysiological analysis

Preparation of hippocampal slices

Hippocampal slices were prepared from 2- to 3-month-old CHL1−/− and age-matched CHL1+/+ mice of both sexes. Mice were anesthetized by CO_2. After immediate decapitation, the brain was removed and cut in two hemispheres. Hemispheres were cut with a Vibroslicer (Leica, Nussloch, Germany) either transversally (for analysis of CA3–CA1, CA3–CA3 and perforant path–dentate gyrus synapses) or horizontally (for perforant path projections to the CA1 and CA3 fields). Based on the characteristics of mossy fiber projections, horizontal slices for mossy fiber–CA3 recordings were cut with an angle of 15°–30° (Eckhardt et al., 2000). Slices were prepared in ice-cold dissection artificial cerebrospinal fluid (ACSF) containing (in mM): 250 sucrose, 25 $NaHCO_3$, 25 glucose, 2.5 KCl, 1.25

NaH_2PO_4, 2 $CaCl_2$, 1 $MgCl_2$ (pH 7.3, adjusted with NaOH). In case of mossy fibers, ACSF was modified to contain (in mM) 230 sucrose, 0.5 $CaCl_2$ and 10 $MgCl_2$ to reduce excitotoxicity. After 350 μm slices were cut, they were kept at room temperature in a large chamber (500 ml) filled with carbogen-bubbled artificial cerebrospinal fluid (ACSF). Recording of field excitatory post-synaptic potentials (fEPSPs) was started 2 h after slice preparations. In the recording chamber, slices were continuously superfused at room temperature with carbogen-bubbled ACSF (2–3 ml/min). Chamber and recording ACSF in contrast to dissection ACSF, contained 125 mM NaCl instead of sucrose.

Analysis of synaptic transmission and plasticity

Field EPSPs (fEPSPs) were recorded by glass pipettes filled with recording ASCF and having a resistance of 2–3 MΩ. Stimulation pulses of 0.2 ms were applied via monopolar stimulating glass electrodes with a broken tip and resistance less then 1 MΩ. The position of electrodes for recording at different synapses is described in below. Basal synaptic transmission was monitored at 0.033 Hz. Responses were amplified and filtered at 1 kHz using CyberAmp 320 (Axon Instruments, Foster City, CA). Data acquisition and analysis were performed using the Pulse program (Heka Electronik, Lambrecht/Pfalz, Germany). Amplitude of responses was measured as a function of intensity of stimulation, and stimulus–response curves in individual experiments were determined with an accuracy of 10 or 20 μA. Amplitude values of 20%, 40%, 60%, 80% and 100% of the maximal (saturated) responses and corresponding stimulus intensities were used for averaging of curves obtained in individual experiments.

Paired-pulse facilitation (PPF) was defined as A2/A1·100%, where A1 and A2 are the amplitudes of the fEPSPs evoked by the first and second pulses, respectively. For most of projections we analyzed PPF with 10, 20, 50, 100 and 200 ms inter-stimulus intervals.

As described below in the Table 1, long-term potentiation (LTP) was induced by different high frequency stimulation (HFS) trains. Each train included 100 pulses delivered at 100 Hz. Inter-train interval was 20 s. Duration of pulses was 0.2 ms. The mean amplitude of fEPSPs recorded 0–10 min before HFS was taken as 100%. The values of LTP were calculated as increase in the mean amplitudes of fEPSPs measured 50–60 min after tetanic stimulation.

For recording of lateral perforant path–dentate gyrus fEPSPs, stimulating and recording electrodes were placed in the outer part of molecular layer of the dentate gyrus (Fig. 5A). Responses were identified by application of paired-pulse stimulation with 50 ms inter-stimulus intervals, and those fEPSPs were used for recording which showed paired-pulse facilitation. LTP was induced by five trains of HFS with inter-train interval of 20 s and 100% of stimulation intensity in disinhibited slices (Evers et al., 2002). Disinhibition by application of 100 μM picrotoxin 10 min before and during HFS is required to induce LTP in perforant path synapses (Hanse and Gustafsson, 1992).

For recording of medial perforant path–dentate gyrus fEPSPs, stimulating and recording electrodes were placed in the inner part of the molecular layer (Fig. 5D) and only those responses were used for recording, which exhibited paired-pulse depression. LTP was induced as in lateral perforant path–dentate gyrus connections.

For recording of direct perforant path–CA3 responses, the stimulating electrode was placed in the stratum lacunosum–moleculare of the CA1 field near to the subiculum and the recording electrode in the stratum lacunosum–moleculare of the CA3 field. To avoid disynaptic activation of CA3 pyramidal neurons, the dentate gyrus was cut with a glass pipette (Fig. 6A) (Berzhanskaya et al., 1998). LTP was induced by two trains of the HFS delivered with 100% of stimulation intensity. Slices were disinhibited with 100 μM picrotoxin.

For recording of direct perforant path–CA1 responses, stimulating and recording electrodes were placed in the stratum lacunosum–moleculare of the CA1 field. The stimulating electrode was as close as possible to the subiculum. Since there is a high possibility to activate perforant path fibers projecting to the dentate gyrus, and therefore cause polysynaptic activation of CA1 neurons via granule cells and CA3 neurons, we cut off the CA3 region (Fig. 6E) (Remondes and Schuman, 2003). LTP was induced by two trains of the HFS delivered with 100% stimulation intensity in disinhibited slices. For disinhibition, 100 μM picrotoxin was applied to the slices 10 min before and during HFS.

For stimulation of associational/commissural projections and recording of CA3–CA3 fEPSPs, one can place the stimulating electrode either in the stratum radiatum of the CA3 field and thus activate associational fibers orthodromically, or place the electrode in the stratum radiatum of the CA1 field and activate associational projections antidromically. We used the second approach (Fig. 7A) to avoid direct stimulation of local CA3 interneurons. Since CA3–CA3 synapses are located in the stratum radiatum of the CA3 field, the recording electrode was placed in this area. LTP was induced by two trains of HFS delivered with 20 s inter-train interval with a stimulation strength being 50% of the supramaximal one (Ito et al., 1997).

For stimulation of Schaffer collateral/commisural projections and recording of CA3–CA1 fEPSPs, the stimulating and recording electrodes were placed in the stratum radiatum of the CA1 region (Fig. 7E). For LTP induction we applied two trains of HFS with the stimulation strength being 50% from the supramaximal one (Muller et al., 1996; Eckhardt et al., 2000).

For recording of mossy fiber–CA3 fEPSPs, the stimulating electrode was placed in the dentate gyrus near to the inner part of the granule cell layer and recording electrode was placed in the stratum lucidum of the CA3 field (Fig. 8A). To avoid polysynaptic responses, a low stimulation strength was used to elicit fEPSPs of 40–60 μV. Mossy fiber responses were identified by their short duration and by frequency-dependent facilitation that was more than 200% elicited in response to 0.33 Hz stimulation (Bukalo et al., 2004). Two trains of HFS were used for induction of LTP, and the interval between trains was 20 s (Eckhardt et al., 2000). Since LTP at this synapse is known to be NMDA receptor-independent, 50 μM AP5 was applied 15 min before and during HFS. To confirm that the recorded field EPSPs were evoked by the stimulation of mossy fibers and were not contaminated by the associational/commissural pathway, an agonist of metabotropic glutamate receptors (2 μM DCG IV) was applied at the end of each experiment and only responses inhibited by >80% were assumed to be elicited by mossy fiber stimulation (Cremer et al., 1998).

Table 1
Summary of stimulation protocols used for induction of LTP at different excitatory hippocampal synapses

Axonal projections	Postsynaptic target	Stimulation strength	Pattern of stimulation	Pharmacology
Medial or lateral perforant paths	DG	100%	5× HFS	Picrotoxin
Perforant path	CA1 or CA3	100%	2× HFS	Picrotoxin
Mossy fibers	CA3	To elicit 40–60 μV responses	2× HFS	AP5
CA3	CA1	50%	2× HFS	No treatment
CA3	CA3	50%	2× HFS	No treatment

DG, dentate gyrus; HFS, high-frequency stimulation.

Statistical analysis

Since in some cases data did not meet the requirements for parametric analysis, behavioral results were analyzed with non-parametric statistical tests. Differences between genotypes were tested with the Mann–Whitney *U*-test. To test dependent data within a genotype the Wilcoxon matched pair test or the Friedman test were used. Since there is no non-parametric test for multi-factorial analysis, a parametric analysis of variance for repeated measurements (ANOVA) having genotype as between-group factor and appropriate between-group factors was applied when required. Newman–Keuls post-hoc analysis was applied when appropriate. Two-way ANOVA for repeated measurements was used to compare stimulus–response curves and paired-pulse facilitation profiles between CHL1+/+ and CHL1−/− mice. *T*-test was used for other comparisons of electrophysiological

parameters. All analyses were two-tailed and significance was set at $p < 0.05$. When not specified, values are presented as mean ± SEM.

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