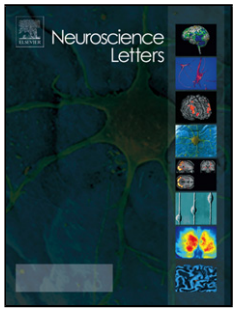


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PII: S0304-3940(16)30655-3
DOI: http://dx.doi.org/doi:10.1016/j.neulet.2016.08.055
Reference: NSL 32271

To appear in: *Neuroscience Letters*

Received date: 27-5-2016
Revised date: 19-7-2016
Accepted date: 30-8-2016

Please cite this article as: Gisela Zalcman, Nicoletta Corbi, Maria Grazia Di Certo, Elisabetta Mattei, Noel Federman, Arturo Romano, Heterozygous Che-1 KO Mice show deficiencies in Object Recognition Memory Persistence, Neuroscience Letters http://dx.doi.org/10.1016/j.neulet.2016.08.055

Title:

# Heterozygous Che-1 KO Mice show deficiencies in Object Recognition Memory Persistence

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Running Title: Che-1 in memory persistence

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**Highlights**

- We generated a genetically modified Che-$1^{+/-}$ KO mice.
- These mice are normal in its locomotor activity and anxiety behavior
- These mice are normal in long-term memory formation.
- Che-$1^{+/-}$ KO mice show deficits in object recognition memory persistence.

ABSTRACT

Transcriptional regulation is a key process in the formation of long-term memories. Che-1 is a protein involved in the regulation of gene transcription that has recently been proved to bind the transcription factor NF-κB, which is known to be involved in many memory-related molecular events. This evidence prompted us to investigate the putative role of Che-1 in memory processes. For this study we newly generated a line of Che-$1^{+/-}$ heterozygous mice. Che-1 homozygous KO mouse is lethal during development, but Che-$1^{+/-}$ heterozygous mouse is normal in its general anatomical and physiological characteristics. We analyzed the behavioral characteristic and memory performance of Che-$1^{+/-}$ mice in two NF-κB dependent types of memory. We found that Che-$1^{+/-}$ mice show similar locomotor activity and thigmotactic behavior than wild type (WT) mice in an open field. In a similar way, no differences were found in anxiety-like behavior between Che-$1^{+/-}$ and WT mice in an elevated plus maze as well as in fear response in a contextual fear conditioning (CFC) and object exploration in a novel object recognition (NOR) task. No differences were found between WT and Che-$1^{+/-}$ mice performance in CFC training and when tested at 24 hours or 7 days after training. Similar performance was found between groups in NOR task, both in training and 24h testing performance. However, we found that object recognition memory

persistence at 7 days was impaired in Che-$1^{+/-}$ heterozygous mice. This is the first evidence showing that Che-1 is involved in memory processes.

Keywords: Che-1, memory persistence, long-term memory, gene transcription, novel object recognition, fear conditioning, mouse

## 1. Introduction

Che-1 was initially identified in humans by two groups as an RNA polymerase II binding protein (Fanciulli et al. 2000), (Lindfors et al. 2000). Che-1 is highly conserved among eukaryotic species. Its rat and mouse homologous proteins are AATF (apoptosis antagonizing transcription factor) and Traube, respectively (Page et al. 1999) (Thomas et al. 2000).

Che-1/AATF/Traube (Che-1) localizes in the nucleus, nucleolus and cytoplasm (Fanciulli et al. 2000, Lindfors et al. 2000, Barbato et al. 2003, Guo and Xie 2004, Ferraris et al. 2012, Hopker et al. 2012) and it is constituvely expressed in the brain, particularly in neurons but not in glia cells (Xu et al, 2013).

Although Che-1 does not bind directly to DNA this protein contains several interaction motifs which have been shown to be required for its interaction with transcriptional activators such as nuclear hormone receptors (Leister et al. 2003), retinoblastoma protein (Fanciulli et al. 2000), STAT3 (Ishigaki et al. 2010) and Nuclear Factor κB subunit, p65 (Bruno et al. 2006), aside of the RNA polymerase II (Fanciulli et al. 2000). All these observations suggest a model in which Che-1 acts as a transactivator or transcription co-factor linking specific transcriptional activators to the general transcription apparatus. As a result of these properties and interactions, Che-1 has been involved in the regulation of the expression of genes that affect cell cycle and

proliferation (Fanciulli et al. 2000, Barbato et al. 2003, Bruno et al. 2006). In neural tissue, it has been shown that Che-1 has an anti-apoptotic activity after traumatic brain injury, during neuronal development and also during neurodegenerative pathologies such as Alzheimer disease, pointing out to a neuroprotective role for this protein (Barbato et al. 2003, Guo and Xie 2004, Di Certo et al. 2007; Xu et al., 2013).

Of particular interest is its binding to p65, an NF-κB subunit, which was first discovered to play a role in inflammatory responses, but which has more recently been proved to be crucial for different cognitive processes such as long-term memory formation and persistence (Engelmann et al. 2014, de la Fuente et al. 2015 for recent reviews). In the central nervous system, p65 nuclear translocation and activity increase after training to different behavioral paradigms such as context-signal memory in crabs (Freudenthal and Romano, 2000), fear conditioning (Lubin and Sweatt 2007, de la Fuente et al. 2014), fear startle potentiation (Yeh et al. 2002), novel object recognition (Federman et al. 2013, Zalcman et al., 2015) and inhibitory avoidance (Freudenthal et al. 2005, Boccia et al. 2007) in mice and rats. Inhibiting this activation impairs long-term memory formation in all of these tasks. Thus, the interaction between Che-1 and NF-κB subunit prompted us to investigate for a possible role of Che-1 in memory formation for two NF-kB dependant tasks: Contextual Fear Conditioning and Novel Object Recognition.

In the present study, we generated a line of Che-$1^{+/-}$ heterozygous mice bearing a null allele with a C57BL/6J genetic background and investigated a possible role for Che-1 in memory formation. Che-1 homozygous KO mouse is lethal during development, but Che-1/wild type (WT) mouse is normal in its general anatomical and physiological features (Thomas et al., 2000).

## 2. Materials and Methods

### *2.1 Animals*

We newly generated a line of Che-$1^{+/-}$ heterozygous mice starting from the $Trb^{gt}$ ES cells gently provided by Thomas (Thomas et al 2000, Desantis et al. 2015). Homozygous KO mice are embrionically lethal at the pre-implantation state. C57BL/6J wild type siblings were used as controls. Male mice, 6-8 weeks old, were used for all the experiments. Animals were housed individually for a week before the experiments began. Water and food was provided *ad libitum* and mice were kept under a 12 h light/dark cycle (lights on at 8:00 A.M.) at a temperature of 21–22°C. Experiments were conducted during the light phase and they were performed in accordance with local regulations and the USA National Institute of Health (NIH) *Guide for the Care and Use of Laboratory Animals* (NIH publication 80-23/96). All procedures were performed with the approval of the Institutional Committee for the Use and Care of Laboratory Animals (CICUAL, protocol number 29) from the University of Buenos Aires. All efforts were made to minimize animal suffering and to reduce the number of animals used. Mice were handled for two days (3min/day) before any behavioral procedure.

### *2.2 Genotypification by PCR analysis.*

For genotypification, a 5 mm sample from the tail of each animal was taken and digested with 300ul of T Solution Buffer (50mM Tris-HCl pH 8, 100mM EDTA, 0.5% SDS) and 100 µg of proteinase K (incubation overnight at 55ºC). The next day DNA was isolated using a phenol-chloroform based DNA extraction protocol, after which the DNA pellet was resuspended in 100 µl of TE buffer (10mM Tris-HCl pH8, 1mM

EDTA). DNA was analyzed by PCR using specific primers to detect the KO allele (Fwd 5'-TTG CCG TAA GTG AAG CGA C-3', Rvs 5'-AGC GGC TGA TGT TGA ACT G-3') which amplified a 400-bp PCR product that was detected on a 1% agarose gel stained with ethidium bromide.

### *2.3 Brain and Spinal Cord Western Blot*

To check Che-1 levels in wild type and genetically modified mice, animals were sacrificed and tissue samples were taken from their brain and spinal cord. Whole-cell lysate protein isolation and purification was done using RIPA Buffer. Total protein in each sample was quantified using the Pierce™ BCA Protein Assay Kit (Thermo Scientific). 20 ug of protein were loaded in a 10% SDS-polyacrilamide gel and run at 110V for 90 min. Proteins were then transferred onto a nitrocellulose membrane (100V, 1h). For western blot analyses, the membrane was incubated with CHE-1 primary antibody (rabbit polyclonal, 1:1000, Fanciulli et al. 2000) and with an antibody for the housekeeping protein Actin (1:1000, mouse monoclonal antibody, Clone AC-40, Sigma Aldrich).

### *2.4 Elevated Plus Maze and Open Field Test*

The *Elevated Plus Maze* (EPM) consisted of two open arms (16 × 5 cm) and two closed arms (16 × 5 × 12 cm) extending from a central platform, which was elevated to a height of 25 cm from the floor. Animals were placed individually at the center of the elevated plus maze with their heads facing towards an open arm and allowed to explore for 5 min. Once the testing session was finished, mice were taken to their homecage and the maze was cleansed with 70% alcohol.

For the *Open Field Test* (OF), mice were placed individually inside an open field arena (30 x 30 cm with 36 cm walls) facing one of the walls (the same for all the animals) and allowed to explore it for 10 min. Once the testing session was finished, mice were taken to their homecages and the open field arena was cleansed with 70% alcohol. Nine squares were drawn on the floor leaving 8 outer or periphery squares and one inner or center square.

For both behavioral tests, the experimental room was conditioned under dim light and animals were taken to the experimental room 30 minutes prior to testing for habituation to the context. The entire session was filmed with a web camera. In the *EPM*, anxiety-like behavior was assessed by scoring *total distance travelled* (cm), *time spent in the open arms* and *time spent in the closed arms*. Spontaneous locomotor activity was evaluated with the *OF Test*, by scoring *total distance traveled* (cm), *mean speed* (cm/s), *number of line crossings* between the nine squares, *time spent in the periphery and time spent in the center.* All these analysis were made using the *ANY-maze* video tracking software (Stoelting co.) except for the *number of line crossings* and the *time spent in the periphery and the center*, which were scored manually.

### *2.5 Contextual Fear Conditioning*

During training session, mice were placed inside a conditioning chamber and after 2 min of acclimatization period they received three foot shocks (0.6mA, 1s) separated 1 min each. After the third shock the animals remained in the chamber for an additional minute and then they were returned to their homecages. The chamber used for this behavioral test is fully described elsewhere (de la Fuente et al. 2011). The

testing session was performed 24 h or 7 days after training, and it consisted in placing the animals inside the conditioning chamber for 5 min in the absence of the footshock.

Training and testing sessions were videotaped to analyze the *freezing* behavior for each animal which is defined as the absence of all movements except those related to breathing and which is commonly used as an index of fear in mice. % Freezing was measured manually by observing if the animal showed a freezing behavior every 5s in a 300 s testing period (60 measurements).

### *2.6 Novel Object Recognition task*

The *Novel Object Recognition* task was carried out as previously described (Zalcman et al, 2015). Mice underwent 3 consecutive days of habituation to the experimental arena, which involved placing each animal inside the experimental arena without object presentation (5min/day). On day 4, the training session took place. Each animal was placed inside the experimental chamber for 10 min with two identical objects. The objects used were 100 ml transparent beakers placed downward or blue blocks (Rasti® toys), both of similar size. The objects were scrubbed with a tissue soaked in 96% alcohol (Sanicol) and then rinsed with bi-distilled water to ensure that no olfactory clues were present. Memory retention was tested 24 h or 7 days after training. During the testing session animals were placed inside the experimental arena and allowed to explore two different objects (a beaker and a rasti block) for 5 min. One of the objects was identical to those explored during the training session (familiar object) and the other one was a novel object. The objects were exposed in the same locations of the chamber as they were in the training session. The location of the novel object was exchanged between left and right for different animals from the same experimental group to avoid place preference during the evaluation session. Training and testing

sessions were filmed with a web camera and exploration behavior was assessed manually as previously described (Zalcman et al. 2015).

During the testing session, the total exploration time for each object was determined and the relative time of novel object exploration was calculated as the discrimination index (DI%) calculated as follows:

*DI%= (t novel- t familiar)/(t novel + t familiar)*100%.*

The mean DI% value was calculated for the different groups of animals. Total times of exploration for each group in training and testing sessions were compared in all of the experiments to verify that there were no differences between groups in this parameter. Animals showing low exploration times (below two standard deviations of the mean time of exploration) were excluded from the experiments.

### *2.7 Statistical Analysis*

All the comparisons between groups was performed with Student *t*-test. Significant differences were considered for p values below 0.05.

## 3. Results

### 3.1 Anxiety and locomotor activity are normal in Che-1$^{+/-}$ mice.

After checking that Che-1 protein expression is reduced in the nervous system of these Che-1$^{+/-}$ animals (Figure 1), we carried out a behavioural characterization of these mice in order to analyze for possible differences in the anxiety levels or locomotor activity in relation to wild type animals. To this aim, we performed elevated plus maze

(EPM) and open field (OF) tasks on Che-$1^{+/-}$ and wild type mice littermates. For the EPM test, animals were placed in the center as indicated on section 2.4 and they were allowed to explore freely for 5min. Behavior on the EPM indicated no differences in anxiety levels, since total distance traveled and time spent in closed and open arms was similar between groups (Figure 1b,c). For the OF test, animals were placed inside an experimental arena and allowed to move freely for 10 min. Figure 1d shows a track example for each group in which it can be noticed that animals explore the whole arena, with a slight preference for the periphery over the center, which is a typical behavior expected for this test. We analyzed different parameters that are affected by locomotor activity effects such as distance traveled (cm), mean speed (cm/s), number of crossings, and time spent in the periphery and the center, and found no significant differences between groups (Figure 1e-g, respectively), suggesting that locomotor activity in mice with decreased Che-1 protein levels is not altered. On the other hand, the time spent in the periphery and in the center, which account for anxiety-related differences, were similar among groups (Figure 1h).

### 3.2 Contextual Fear conditioning memory is not impaired in Che$^{+/-}$ mice.

We decided to investigate if Che-$1^{+/-}$ mice could have memory deficits in a *contextual fear conditioning* (CFC) task. CFC involves an association between an aversive unconditioned stimulus and contextual features of the place.

CFC experimental design is shown on Figure 2a and its procedure is described on section 2.5. During the training session no statistical differences in % freezing were observed between groups (% freezing pre-shock: WT = 14,35 ± 4,40, Che-$1^{+/-}$ = 12,50 ± 4,00; % freezing post-shock: WT = 68,52 ± 4,34, Che-$1^{+/-}$ = 65,60 ± 2,49). Memory

retention was tested at 24 h or 7 days later using two independent sets of WT and Che-$1^{+/-}$ animals. Percentage of freezing values for Che-$1^{+/-}$ mice and WT groups determined 24 h after training (Figure 2b) showed memory retention in both groups without statistical differences. Furthermore, testing on day 7 showed similar levels of retention (Figure 2c), indicating that the training protocol induced a strong-long lasting memory which was similar for both groups. Taken together, these results suggest that this type of aversive memory is not affected in Che-$1^{+/-}$ mice.

### 3.3 Object Recognition long-term memory persistence but not its formation is impaired in Che$^{+/-}$ mice.

Novel Object Recognition (NOR) involves a neutral experience which takes advantage of the natural preference that mice have for exploring novel items over familiar ones to measure spontaneous object recognition memory. The term "spontaneous" denotes that no reinforcement or punishment was used to induce object exploration. NOR task was carried out as described in section 2.6. Briefly, in the training session animals were placed inside an experimental chamber with two identical objects and they were allowed to explore them for 10 min. 24h or 7 days after, memory retention was assessed with a 5 min testing session in which animals were placed inside the experimental arena containing two different objects, one was the previously experienced object and the other one was novel (Figure 3a). Figure 3b shows that exploration times during the training session were similar between groups for both experiments, indicating that motivation to explore the objects was unaffected in Che-$1^{+/-}$ animals. During the testing session, because rodents have a natural preference for novel objects over familiar ones, we expected animals with good memory retention to spend more time exploring the novel object. To account for the difference in the exploration

time for each object, we calculated a discrimination index as indicated in section 2.6. %DI between Che-$1^{+/-}$ and WT animals showed no significant differences when tested 24 h after training (Figure 3c). However, a significant decrease in %DI was found for the genetically modified animals in comparison to WT animals when tested 7 days after training (Figure 3d). This result indicates that Che-$1^{+/-}$ animals are specifically impaired in the persistence but not the formation of NOR memory.

## 4. Discussion

Over all, our results suggest that Che-1 is particularly involved in the molecular mechanisms of object recognition memory persistence but not in the initial formation of this memory or in memory for a fear conditioned task. The different outcome seen between the neutral and the aversive task is noteworthy. However, we cannot rule out a ceiling effect in the behavioral response of the mice in CFC impeding us to detect differences in the level of freezing at 7 days. A less strong training protocol could reveal an impairment of memory persistence in CFC as previously found in NOR.

The EPM and OF results indicate that locomotor activity and anxiety behavior are similar between Che-1 deficient mice and the wild type control animals. Furthermore, freezing behavior and motivation to explore objects showed no significant differences between groups. Thus we introduced newly generated transgenic mice which are suitable for further investigations on the role of Che-1 in memory formation, persistence and other mnemonic processes such as reconsolidation and extinction, as well as in different behavioral paradigms. We did not test the effect of Che-1 deficits on short term memory because Che-1 is mainly involved in gene expression changes and short-term memory is independent of this molecular process (Goelet et al., 1986).

Moreover, if short-term memory (STM) was impaired, we would expect to find impairment in long-term memory (LTM), which we did not observe.

Regarding of the subtle effect found in the process of NOR memory persistence and caused by the lower level of Che-1 in the genetically modified mice, we propose two molecular mechanisms in which Che-1 could be taking part. It was recently found that NF-κB-dependent epigenetic mechanisms are involved in memory persistence. In fact, histone acetylation was increased after strong training in a NOR task and this process was mostly dependent on NF-κB and specifically necessary for memory persistence (Federman et al. 2013). In relation to this, phosphorylated Che-1 was found to bind to p65 NF-κB subunit (Bruno et al., 2006) and to compete with HDAC1 binding to different gene promoters (Bruno et al. 2002), thus affecting the acetylation/deacetylation balance in neurons. Interestingly, a recent study showed that HDAC1 is involved in memory-related processes (Bahari-Javan et al., 2012). On the other hand, Che-1 has also been involved in cell growth and cell fate events. Most of the work done with Che-1 protein is linked to cancer progression and even though there is not a clear consensus regarding Che-1 function, an important part of such evidence points to an anti-apoptotic effect on carcinogenic cell lines and tissue (Iezzi and Fanciulli 2015, for a recent review). Thus, from this evidence we speculate that Che-1 could be involved in memory persistence either by affecting the epigenetic regulation of gene transcription involved in this process or through the regulation of apoptotic events in neuronal progenitors, such as the immature granule cells of the hippocampus. Ongoing experiments are aimed at studying learning-induced Che-1 phosphorylation, and Che-1-dependent histone acetylation as well as NF-kB activation. These changes may be taking place at key structures involved in NOR, such as hippocampus, perirhinal

cortex and medial prefrontal cortex (Brown and Aggleton, 2001; Barker et al., 2007; Cohen and Stackman, 2015).

## 5. Conclussions

Che-1+/- mice show normal anxiety and locomotor activity compared to wild type animals. Long-term memory formation and persistence for an aversive task such as contextual fear conditioning is not impaired, but long term memory persistence for a neutral task such as novel object recognition is specifically impaired while its formation is left intact.

**ACKNOWLEDGEMENTS.**

We thank Dr. Claudio Passananti, Dr. Maurizio Fanciulli and Dr. Liliana Orelli for helpful comments on the manuscript. This work was supported by research grants from the National Agency of Scientific and Technological Promotion of Argentina (ANPCyT) PICT1482 and PICT2369, National Council of Research (CONICET) PIP5466 and University of Buenos Aires grant X198.

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## FIGURE LEGENDS

***Figure 1. Anxiety levels and locomotor activity are similar between wild type and Che-1$^{+/-}$ mice.***

***a.*** *Che-1 protein levels are reduced in the brain and spinal cord of Che-1$^{+/-}$ mice.* ***b,c****. Distance traveled (b) and time spent in closed and open arms (c) of an elevated plus-maze ($n_{WT}$= 9, $n_{Che\text{-}1+/-}$ =7).* ***d.*** *Representative tracks for the WT and Che-1$^{+/-}$ mice, respectively****. e-h****. Distance traveled (e), mean speed (f), total number of crossings (g) and time spent in the periphery and center (h) in an open field test. ($n_{wt}$=10, $n_{Che\text{-}1+/-}$=8). All data are shown as mean ± s.e.m. No statistical differences were found between WT and Che-1$^{+/-}$ groups in Student t-test (EPM: distance traveled t=0.04874 df=14 p=0.9618, time spent in closed arms t=0.08787 df=14, p=0.9312, time in open arms t=0.2225 df=14 p=0.8271; OF: distance traveled t=0.5408 df=15 p=0.5966, mean speed t=0.04778 df=15 p=0.9625, total number of crossings t=0.07256 df=15 p=0.9431, time spent in the periphery and center t=1.260 df=15 p=0.2269 and t=0.2453 df=15 p=0.8098, respectively)*

***Figure 2. Fear conditioning memory is not affected in Che-1$^{+/-}$ heterozygous mice.***

***a.*** *Diagram outlining the experiment.* ***b,c.*** *Graph showing % freezing 24hs ($n_{WT}$= 11, $n_{Che\text{-}1}^{+/-}$=11) (b) and 7 days ($n_{WT}$= 9, $n_{Che\text{-}1}^{+/-}$=12) (c) after contextual fear conditioning training. All data are shown as mean ± s.e.m. No statistical differences were found between WT and Che-1$^{+/-}$ groups in Student t-test (%freezing 24hs: t=0.2892 df= 20 p=0.7754; %freezing 7 days: t=1.305 df=19 p=0.2076).*

***Figure 3. 7 day but not 24h retention of object recognition memory is impaired in Che-1$^{+/-}$ heterozygous mice.***

***a.*** *Diagram showing a representation of NOR training and testing session.* ***b.*** *Total time of object exploration during the training session.* ***c.*** *Discrimination index (%DI) 24 h after training.* ***d****. % DI 7 days after training. $n_{WT,\,24hs}$= 9, $n_{Che\text{-}1}^{+/-}{}_{,\,24hs}$ =7; $n_{WT,\,7d}$= 10,*

*$n_{Che\text{-}1^{+/-},\,7d}$ =12. All data are shown as mean ± s.e.m.* stands for significant differences in Student t-test, p<0.05 (total time of exploration 24hs t=0.02827 df=14 p=0.9779, total time of exploration 7days t=0.1551 df=20 p=0.8793; $\%DI_{24hs}$ t=0.08082 df=14 p=0.9367, $\%DI_{7\ days}$: t=2.234 df=20 p= 0.0370).*

**Figure 1**

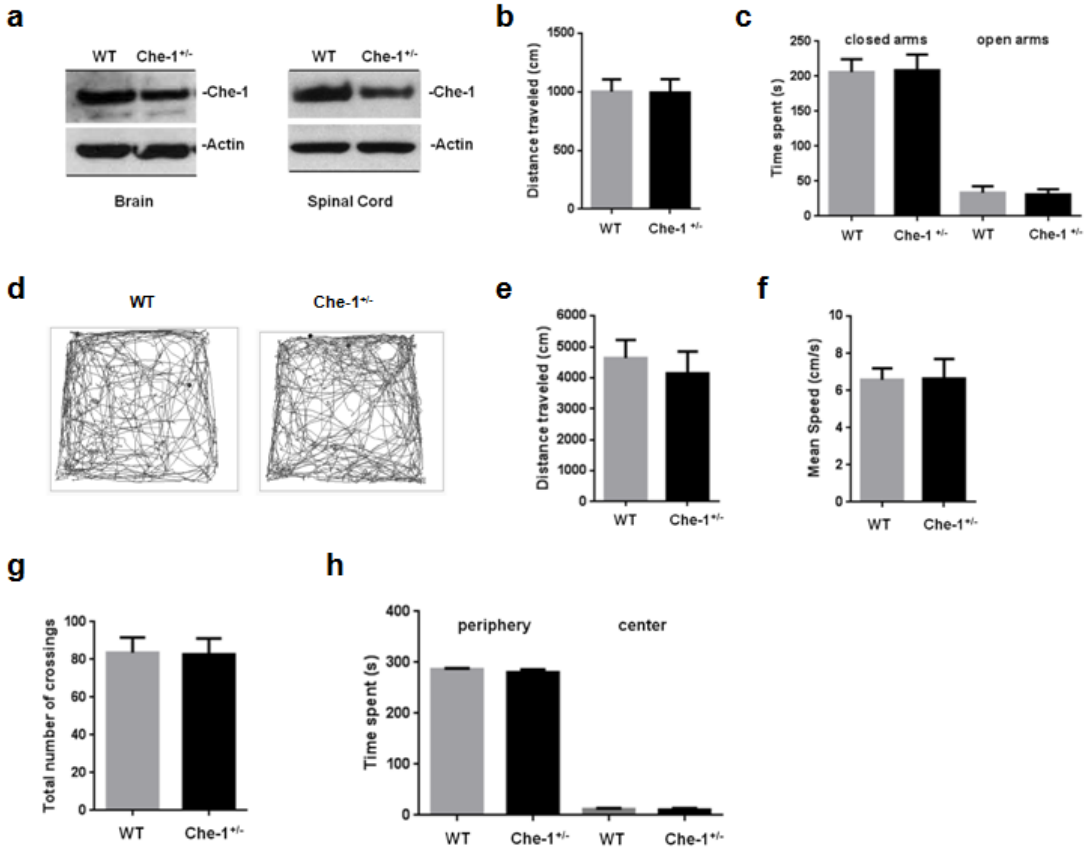

**Figure 2**

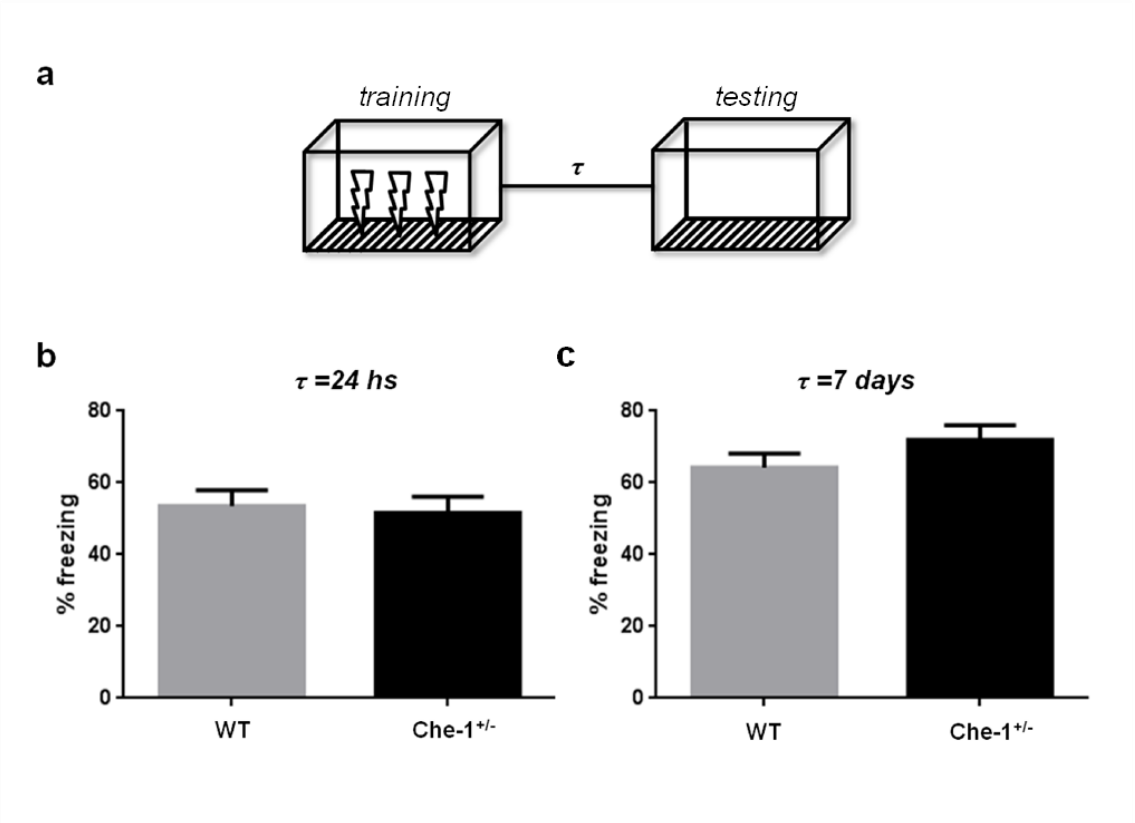

**Figure 3**