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Wip1 phosphatase positively modulates dendritic spine morphology and memory processes through the p38MAPK signaling pathway

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Abstract

Dendritic spine morphology is modulated by protein kinase p38, a mitogen-activated protein (MAPK), in the hippocampus. Protein p38MAPK is a substrate of wip1, a protein phosphatase. The role of wip1 in the central nervous system (CNS) has never been explored. Here, we report a novel function of wip1 in dendritic spine morphology and memory processes. Wip1 deficiency decreases dendritic spine size and density in pyramidal neurons of the hippocampal CA1 region. Simultaneously, impairments in object recognition tasks and contextual memory occur in wip1 deficient mice, but are reversed in wip1/p38 double mutant mice. Thus, our findings demonstrate that wip1 modulates dendritic morphology and memory processes through the p38MAPK signaling pathway. In addition to the well-characterized role of the wip1/p38MAPK in cell death and differentiation, we revealed the novel contribution of wip1 to cognition and dendritic spine morphology, which may suggest new approaches to treating neurodegenerative disorders.

Keywords

morphology, spine, dendritic, pathway, modulates, processes, positively, signaling, phosphatase, wip1, p38mapk, memory

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Wip1 phosphatase positively modulates dendritic spine morphology and memory processes through the p38MAPK signaling pathway

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Keywords: Wip1 phosphatase, p38MAPK, memory, dendritic spine morphology, hippocampus, signaling pathway

Dendritic spine morphology is modulated by protein kinase p38, a mitogen-activated protein (MAPK), in the hippocampus. Protein p38MAPK is a substrate of wip1, a protein phosphatase. The role of wip1 in the central nervous system (CNS) has never been explored. Here, we report a novel function of wip1 in dendritic spine morphology and memory processes. Wip1 deficiency decreases dendritic spine size and density in pyramidal neurons of the hippocampal CA1 region. Simultaneously, impairments in object recognition tasks and contextual memory occur in wip1 deficient mice, but are reversed in wip1/p38 double mutant mice. Thus, our findings demonstrate that wip1 modulates dendritic morphology and memory processes through the p38MAPK signaling pathway. In addition to the well-characterized role of the wip1/p38MAPK in cell death and differentiation, we revealed the novel contribution of wip1 to cognition and dendritic spine morphology, which may suggest new approaches to treating neurodegenerative disorders.

Introduction

Dendritic spines are the main excitatory sites of the neurons in brain, undergoing morphological changes in response to neuronal activity.^{1–3} Because of this continual dynamic, an increase in the size and number of dendritic spines might represent a physical substrate for learning and memory processes.^{1,3} However, these molecular mechanisms underlying learning and memory processes and modulation of dendritic spine structure, are not fully understood.

Several molecular families, including scaffold proteins, and regulators of the cytoskeleton, have been found involved in the regulation of dendritic spine morphology and density. In particular, several protein kinases, such as mitogen-activated protein kinase (MAPK), calcium/calmodulin-dependent protein kinase and protein phosphatases (PPs) (PP1 and PP2) present in the post-synaptic compartment, are essential factors in the regulation of dendritic spine morphology^{4–6} and synaptic plasticity.^{4,7,8} In addition, PPs have been previously associated with Alzheimer disease (AD), which is the most common cause of memory deficits and dementia.^{9,10}

Wip1, encoded by the *PPM1D* gene, is part of the PP2C family of protein serine-threonine phosphatases.¹¹ To date, three dephosphorylation targets of wip1 [p53, p38MAPK and ataxia-telangiectasia mutated (ATM)] have been reported.^{12–15} For p38MAPK, wip1 dephosphorylates an activating threonine residue that is essential for p38MAPK activity.¹⁶ Wip1 expression is induced in a p53-dependent manner following gamma and UV radiation.^{11,16} Although wip1 mRNA was found in mouse brain,¹² the roles of wip1 in the CNS, particularly in dendritic spine morphology and/or memory processes, have never been explored.

p38MAPK, located in postsynaptic dendrites, has been implicated in cytoskeleton reorganisation.^{17–19} Inhibition of p38MAPK activity leads to an increase in the size of dendritic spines,²⁰ which implicates p38MAPK in the regulation of the morphology of dendritic spines. Given that p38MAPK is a downstream element of wip1 signaling, it might be hypothesized that wip1 would have functions in CNS neurons. Here, we have studied novel roles of wip1 in the modulation of dendritic spine morphology and learning and memory processes through the p38MAPK pathway in the CNS.

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Results

Role of wip1 protein in modulating dendritic spine morphology.

Dendritic spine morphology was examined by Golgi staining, performed on hippocampal sections from adult *wip1^{+/+}* and *wip1^{-/-}* mice. The staining revealed significant differences between *wip1^{+/+}* and *wip1^{-/-}* mice in terms of dendritic morphology (Fig. 1A and B). Measurements of spine length, spine head width and spine density were performed on Golgi stained neurons in CA1 of the hippocampus. Our results showed that spine length was significantly reduced in *wip1^{-/-}* ($0.79 \pm 0.02 \mu\text{m}$) vs. *wip1^{+/+}* neurons ($0.89 \pm 0.05 \mu\text{m}$; $p = 0.04$; Fig. 1). Spine head widths were also significantly reduced in *wip1^{-/-}* hippocampal neurons ($0.41 \pm 0.03 \mu\text{m}$) compared with the ones measured in *wip1^{+/+}*

hippocampal neurons ($0.5 \pm 0.07 \mu\text{m}$; $p = 0.023$; Fig. 1Bb). The spine density measured in CA1 of *wip1^{+/+}* mice (17.7 ± 3.77) was more than 70% greater than in *wip1^{-/-}* mice (6.7 ± 0.5 ; $p = 0.0012$; Fig. 1Bc). The spine length ($0.8 \pm 0.01 \mu\text{m}$) and the spine head width ($0.38 \pm 0.05 \mu\text{m}$) were significantly reduced in *wip1^{-/-}* mice compared with *wip1^{+/+}* mice ($p = 0.048$ and $p = 0.02$, respectively) (Fig. 1Ba and b). Regarding spine density (number of spines per $10 \mu\text{m}$ length of dendrites), the significant decrease (35%) reported in *wip1^{-/-}* mice compared with control mice ($11.4 \pm 2.06 \mu\text{m}$, $p = 0.012$) was lower than that observed in the *wip1^{-/-}* mice (62%, $p = 0.0012$) (Fig. 1Bc). These data were confirmed by the immunostaining of the actin filaments with rhodamine-phalloidin in the hippocampal neurons from *wip1^{+/+}*, *wip1^{+/-}* and *wip1^{-/-}* mice (Fig. S1). Quantification

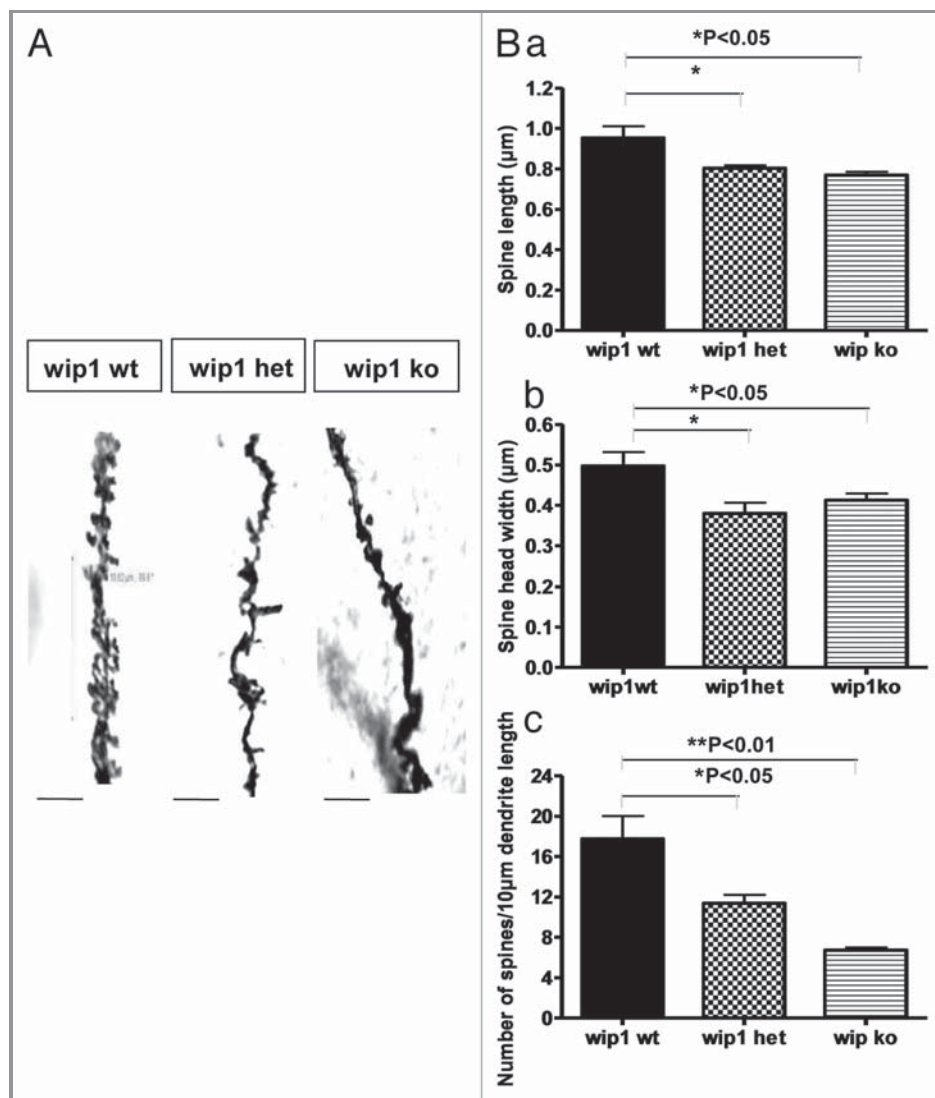


Figure 1. Morphological analysis of the dendrites in CA1 of the hippocampus in *wip1 wt* and *wip1 ko* mice. (A) Golgi staining of dendrites of adult pyramidal neurons in CA1 of the hippocampus of *wip wt*, *wip1 het* and *wip1 ko* mice. Representative images of dendritic fragments from *wip1 wt*, *wip1 het* and *wip1 ko* mice. (B) Quantification of spine length (a), spine head width (b) and spine density (c) of the Golgi stained neurons in *wip1 wt* ($n = 9$), *wip1 het* ($n = 4$) and *wip1 ko* mice ($n = 8$). * $p < 0.05$, ** $p < 0.01$. Bars = $4 \mu\text{m}$.

showed that spine lengths in *wip1*^{-/-} (1.35 ± 0.06 μm) and in *wip1*^{+/-} (1.84 ± 0.16 μm) hippocampal cells were significantly reduced compared with the spine lengths in *wip1*^{+/+} neurons (2.23 ± 0.15 μm). The spine densities of *wip1*^{+/-} (1.7 ± 0.2) and *wip1*^{-/-} (1.9 ± 0.5) neurons were also significantly decreased compared with the spine density measured in *wip1*^{+/+} cells (2.4 ± 0.8). However, spine head width did not differ significantly. Interestingly, branching indexes (number of branch points divided by average dendritic length) in *wip1*^{+/-} (0.54 ± 0.07) and *wip1*^{-/-} (0.44 ± 0.05) neurons were significantly reduced, compared with *wip1*^{+/+} mice (1.02 ± 0.05). Altogether, these data demonstrate, for the first time, that *wip1* modulates dendritic spine morphology in hippocampus.

Wip1 deficiency impairs object recognition task and contextual memory. As *wip1* plays a role in the modulation of dendritic spine morphology, we hypothesized that *wip1* might be involved in learning and memory processes. We therefore performed three behavioral tests: an object recognition test to evaluate associative memory,²¹ contextual fear conditioning²² and a T-maze spontaneous alternation task.²³

Recognition memory tasks have previously been shown to be hippocampal-dependent in lesion studies in both rodents²⁴⁻²⁶ and primates.^{27,28} To assess whether *wip1* affects associative memory, the object recognition test was performed with *wip1*^{+/+}, *wip1*^{+/-} and *wip1*^{-/-} mice. During the training session of this test, the percentage of time spent exploring the identical objects in the open-field was not significantly different among *wip1*^{+/+} (19.4%), *wip1*^{+/-} (17.8%) and *wip1*^{-/-} (20.7%; n = 8–11) mice. Nevertheless, both *wip1*^{+/-} and *wip1*^{-/-} mice showed a significantly higher recognition index (60.3% and 59.6%, respectively) compared with *wip1*^{-/-} mice (49.6%, p = 0.0013 and p = 0.0186 respectively; Fig. 2). With a recognition index inferior to 50%, the mice deficient in *wip1* showed an inability to remember the familiar object previously explored in the training session and they spent less time exploring the new object, suggesting that *wip1*

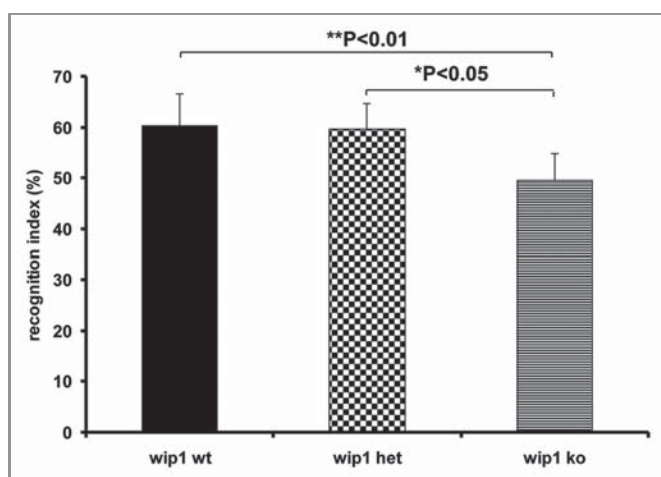


Figure 2. Evaluation of associative memory in *wip1* wt, *wip1* het and *wip1* ko mice. Evaluation of memory by object recognition test in *wip1* wt (n = 11), *wip1* het (n = 8) and *wip1* ko (n = 9) mice. Values of recognition index are means ± SEM. *: p < 0.05, **: p < 0.01.

deficient mice have impaired memory in the object recognition test.

In order to further explore the differences in memory between *wip1*^{+/+} and *wip1*^{-/-} mice, animals were tested on contextual fear conditioning and T-maze spontaneous alternation tasks (Fig. 3A–C). Both of these behavioral tasks have previously been reported involving the hippocampal region.²⁹⁻³²

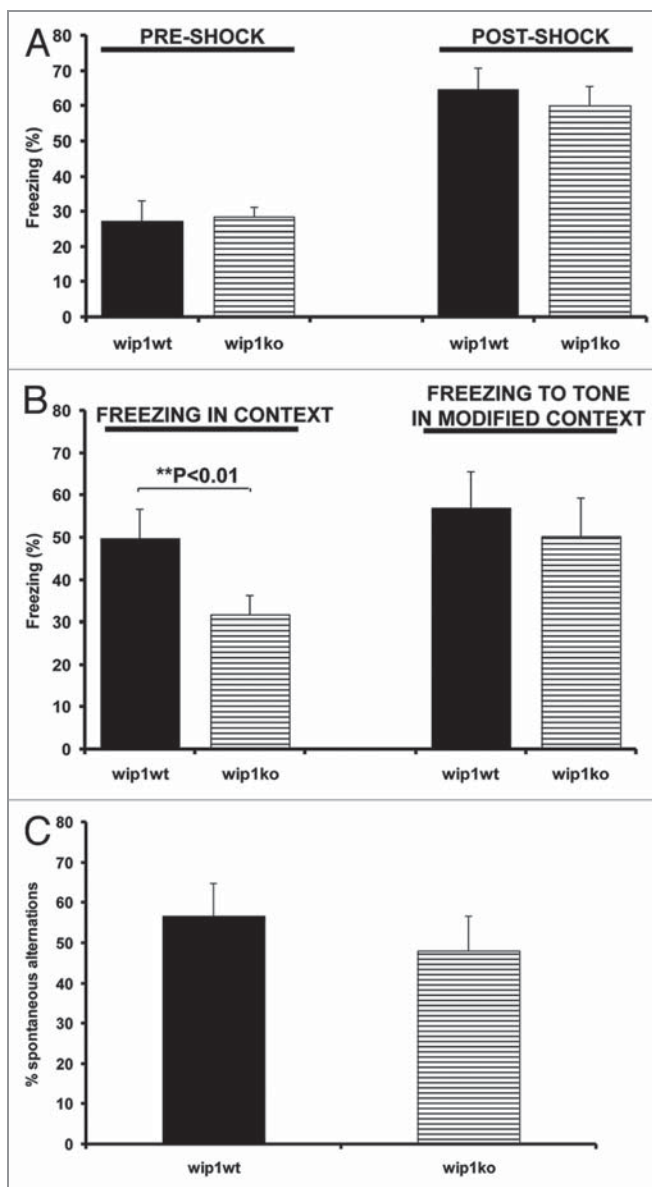


Figure 3. Evaluation of contextual memory and T-maze test in *wip1* wt and *wip1* ko mice. (A) Freezing response of *wip1* wt (n = 11) and *wip1* ko (n = 9) mice during fear conditioning trials (tone + shock conditioning). Values of freezing are expressed as mean ± SEM. (B) Freezing response of *wip1* wt and *wip1* ko mice to contextual exposure and to presentation of conditioned stimulus (tone) in a modified context 24 h following fear conditioning. Values of freezing are expressed as mean ± SEM **: p < 0.01. (C) Spontaneous alternation tasks on the T-maze for *wip1* wt (n = 11) and *wip1* ko (n = 9). Values of spontaneous alternation are expressed as mean ± SEM.

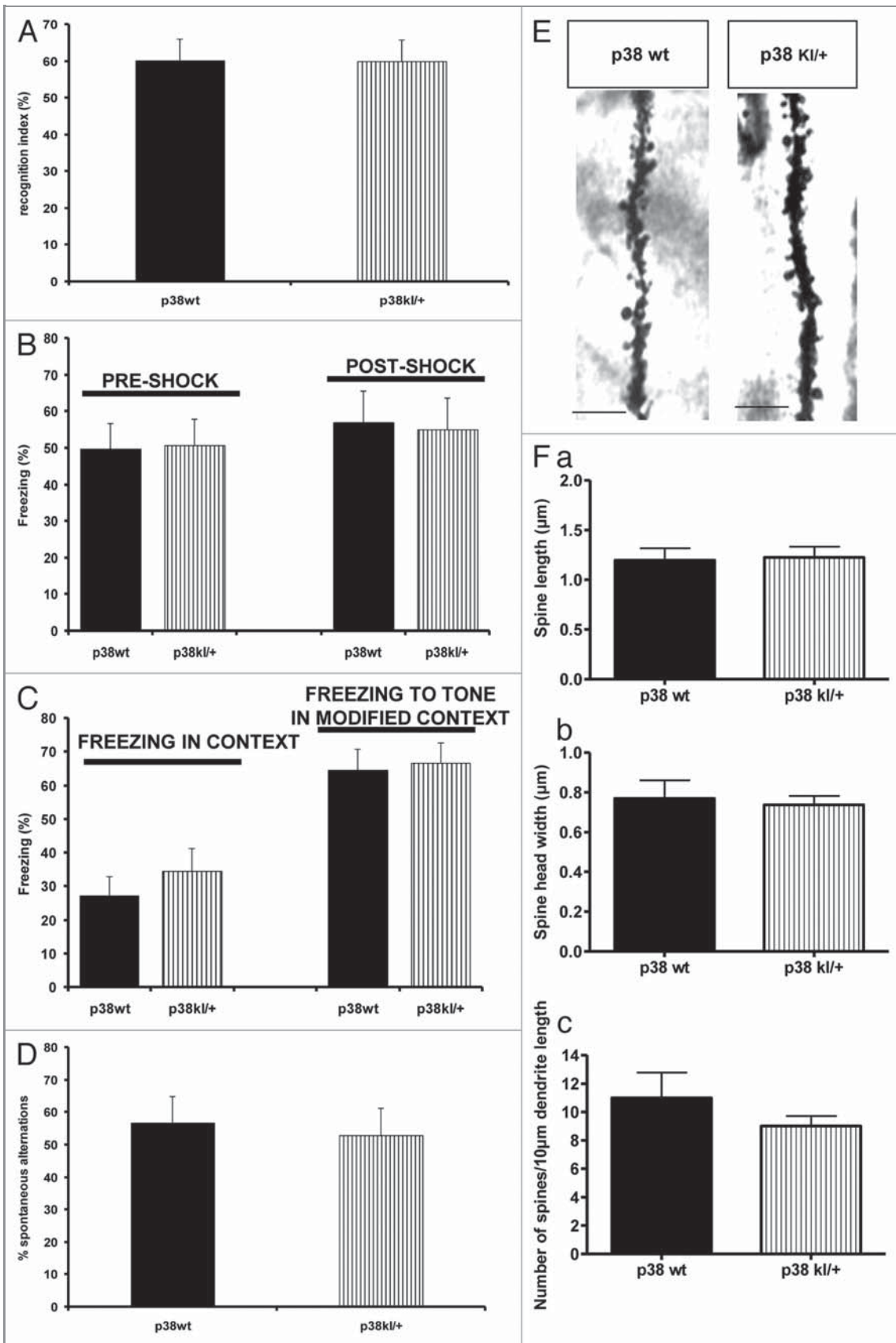


Figure 4 (See opposite page). Behavioral tests and dendrite spine morphology in *p38* wt and *p38^{KO/+}* mice. (A) Evaluation of memory by object recognition test in *p38* wt (n = 11) and *p38^{KO/+}* (n = 9) mice. Recognition index values are shown as mean ± SEM. (B) Freezing response of *p38* wt (n = 11) and *p38^{KO/+}* (n = 9) mice during fear conditioning trials (tone + shock conditioning). Values of freezing are expressed as mean ± SEM. (C) Freezing response of *p38* wt and *p38^{KO/+}* mice to contextual exposure and to presentation of conditioned stimulus (tone) in a modified context 24 h following fear conditioning. Values of freezing are expressed as mean ± SEM. (D) T-maze spontaneous alternation for *p38* wt (n = 11) and *p38^{KO/+}* (n = 9). Values of spontaneous alternation are expressed as mean ± SEM. (E) Golgi staining of dendrites of adult pyramidal neurons in CA1 hippocampus *p38* wt and *p38^{KO/+}* mice. Representative images of dendritic fragments from *p38* wt and *p38^{KO/+}* mice. (F) Quantification of spine length (a), spine head width (b), and spine density (c) of Golgi stained neurons in *p38* wt (n = 8) and *p38^{KO/+}* mice (n = 7). Bars = 4 μm.

In the training trial of the contextual fear conditioning task, mice were placed in the test chamber and subsequently received two weak footshocks. Before the footshocks were delivered, the *wip1^{+/+}* and *wip1^{-/-}* mice explored the conditioning chamber with no significant difference in their mobility (Fig. 3A, $p > 0.05$). Both groups of mice displayed a comparable increase in freezing while the footshocks were administered, demonstrating that these mice do not have a sensorimotor performance deficit, such as an inability to freeze (Fig. 3A, $p > 0.05$). When returned to the conditioning chamber on the next day, the *wip1^{+/+}* mice showed a greater conditioned freezing response ($49.78 \pm 6.9\%$) than *wip1* deficient mice ($31.6 \pm 4.5\%$) (Fig. 3B, $p = 0.0012$). In contrast to this impaired contextual memory, *wip1^{-/-}* mice were not significantly impaired in the different context of a modified chamber, where they displayed similar levels of conditioned freezing to the tone (CS) as the *wip1^{+/+}* mice (Fig. 3B, $p > 0.05$). Thus the deficit in contextual fear conditioning was not due to a general impairment in freezing ability but was more likely related to a cognitive deficit.³³ Thus, in this hippocampal-dependent task, *wip1* deficient mice showed impairment, as they did in the object recognition task.

When we assessed performance in a T-maze spontaneous alternation task measuring exploratory behavior as well as working memory, there was no significant difference between *wip1^{+/+}* and *wip1^{-/-}* mice for the measurement of the percentage of spontaneous alternation (Fig. 3C, $p = 0.06$). Nevertheless, *wip1* deficient mice had a smaller percentage of spontaneous alternation ($48.06 \pm 8.6\%$) than *wip1* wild-type mice ($56.66 \pm 8.3\%$) (Fig. 3C). This result was not due to a difference in completed trials, *wip1^{-/-}* mice having completed on average 8.38 ± 1.2 trials of the 10 trials compared with the 8.63 ± 0.9 trials completed by the control mice.

Together with the impairments in dendritic spine morphology, the behavioral data further demonstrates that *wip1* has functions in the CNS.

***p38* deficiency maintains novel object recognition and contextual fear conditioning memory and normal dendritic spine morphology.** To investigate the potential role of *p38*MAPK in memory, we measured novel object recognition test performance, contextual fear conditioning and the dendritic spine morphology in *p38^{KO/+}* and *p38^{+/+}* littermates.

In the novel object recognition task, the training session revealed a comparable percentage of time spent exploring the object in both groups of mice (n = 8; 20.4% and 17.5% for *p38^{+/+}* and *p38^{KO/+}* mice respectively). During the test, the *p38^{+/+}* mice (60.1%) showed no difference in recognition index compared with the *p38^{KO/+}* mice (59.9%, $p > 0.05$; Fig. 4A). Both groups of

mice tested presented a recognition index superior to 50%, suggesting that the memory in these mice was not affected by the deficiency in the *p38*MAPK gene. In the contextual fear conditioning test there was significant difference in the mobility of *p38^{+/+}* and *p38^{KO/+}* mice before and after the footshocks during the conditioning phase (Fig. 4B, $p > 0.05$). The *p38^{+/+}* and *p38^{KO/+}* mice displayed a similar percentage of freezing in context (49.8% and 50.7% respectively) and freezing to tone in a modified context (56.9% and 55% respectively) (Fig. 4C, $p > 0.05$). Together these data show that memory in the novel object recognition test, contextual fear conditioning test and T-maze spontaneous alternation test (Fig. 4D) was similar in *p38^{KO/+}* mice and control mice.

Next, we further analyzed spine morphology in CA1 of the hippocampus. Golgi staining did not reveal any significant differences between *p38^{KO/+}* and *p38^{+/+}* mice in terms of dendritic spine morphology as measured by the three parameters analyzed ($p > 0.05$; Fig. 4E and F). These results indicate that, in contrast to *wip1*, mutant *p38*MAPK deficient mice showed no impairment in ability to acquire associative and contextual memory tasks and also a preservation of normal dendritic spine morphology in the hippocampus.

***Wip1* deficiency associated impairments in dendritic spine morphology, and memory are reversed in *wip1^{-/-}p38^{KO/+}* double mutant mice.** The previous results indicate that *p38*MAPK is a downstream element of *wip1* signaling in modulation of dendritic spine morphology and memory processes. To further confirm this notion, we investigated dendritic spines in CA1 of the hippocampus and memory in the novel object recognition test, contextual fear conditioning test and T-maze spontaneous alternation procedure in *wip1^{-/-}p38^{KO/+}* and *wip1^{+/+}p38^{KO/+}* littermates. Analysis of spine morphology (Fig. 5A and B) showed no significant differences in spine length and spine head widths between neurons from *wip1^{+/+}p38^{+/+}* (1.17 ± 0.10 μm and 0.56 ± 0.08 μm, respectively) and *wip1^{-/-}p38^{KO/+}* (0.99 ± 0.08 μm and 0.58 ± 0.06 μm, respectively) mice ($p > 0.05$; Fig. 5Ba and b). However, the spine density in *wip1^{-/-}p38^{KO/+}* mice (7.4 ± 0.67) was significantly decreased by about 50% compared with their *wip1^{+/+}p38^{+/+}* littermates (15.6 ± 1.7 ; $p = 0.002$; Fig. 5Bc).

The reduction in dendritic spine density was higher in *wip1* deficient mice (about 70%) than in double mutant mice (about 50%), suggesting that *wip1* modulates spine morphology through the *p38*MAPK signaling pathway but not necessarily exclusively through this pathway.

The novel object recognition test showed that there was no difference in the recognition index between *wip1^{-/-}p38^{KO/+}* (57.8%) and *wip1^{+/+}p38^{+/+}* mice (60.1%, $p > 0.05$) (Fig. 5C).

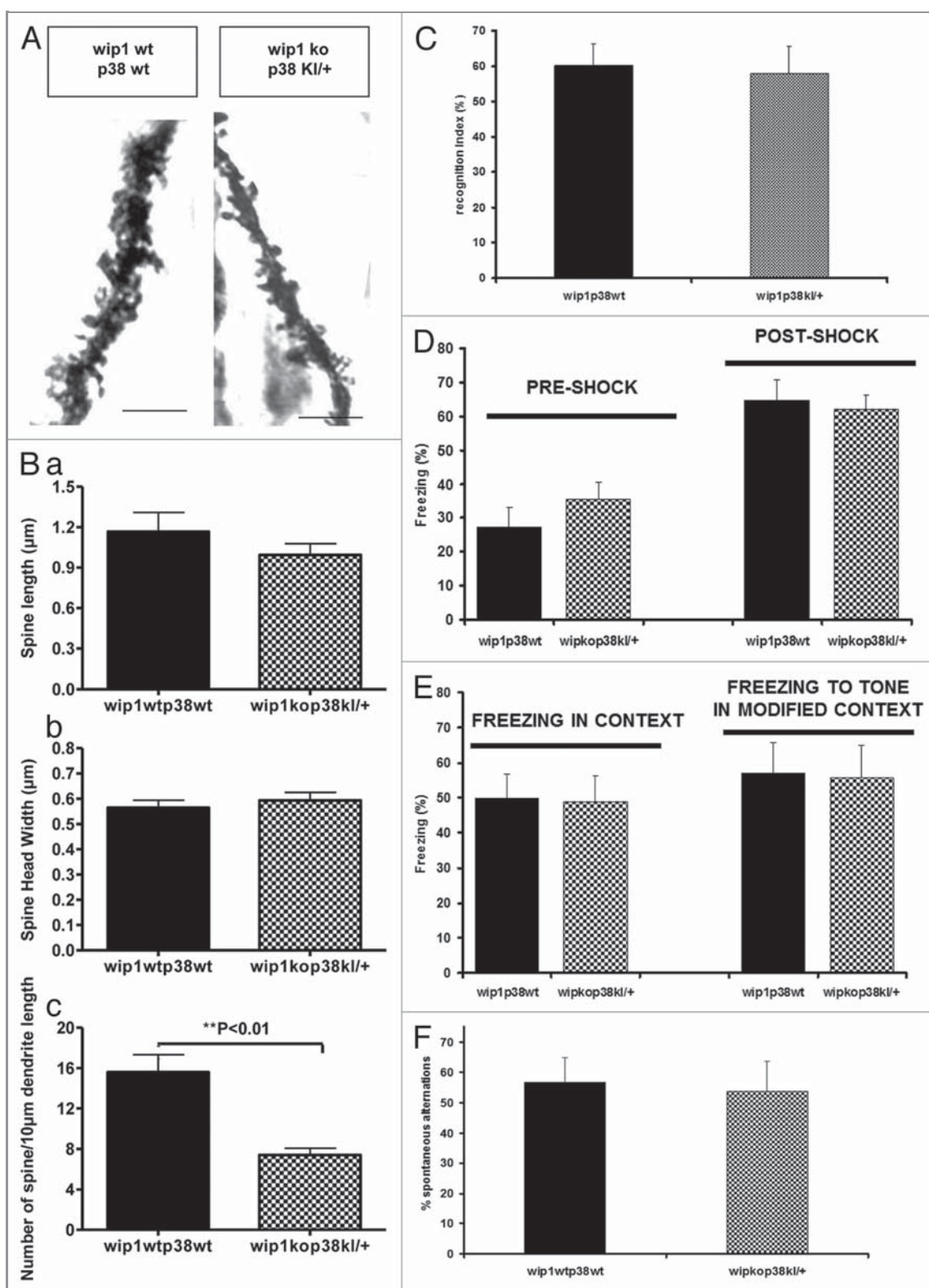


Figure 5. For figure legend, see page 339.

Figure 5 (See opposite page). Wip1 deficiency-associated changes in dendritic spine morphology and behavioral tests are reversed in the *wip1kop38^{KI/+}* double mutant mice. (A) Golgi staining of dendrites of adult pyramidal neurons in CA1 hippocampus in *wip1 wtp38wt* and *wip1kop38^{KI/+}* mice. Representative pictures of dendritic fragments from *wip1wtp38wt* and *wip1kop38^{KI/+}* mice. (B) Quantification of spine length (a), spine head width (b), and spine density (c) of the Golgi stained neurons in *wip1wtp38wt* (n = 7) and *wip1ko p38^{KI/+}* mice (n = 6). Bars = 4 μ m. (C) Evaluation of memory by object recognition test in *wip1wtp38wt* (n = 11) and *wip1kop38^{KI/+}* (n = 8) mice. Recognition index values are reported as mean \pm SEM. (D) Freezing response of *wip1wtp38wt* (n = 11) and *wip1kop38^{KI/+}* (n = 8) mice during fear conditioning trials (tone + shock conditioning). Values of freezing are expressed as mean \pm SEM. (E) Freezing response of *wip1wtp38wt* and *wip1kop38^{KI/+}* mice to contextual exposure and to presentation of conditioned stimulus (tone) in a modified context 24 h following fear conditioning. Values of freezing are expressed as mean \pm SEM. (F) T-maze spontaneous alternation in *wip1wtp38wt* (n = 11) and *wip1kop38^{KI/+}* (n = 8). Values of spontaneous alternation are expressed as mean \pm SEM.

Likewise, the contextual fear test showed no significant difference in the percentage of freezing measured in *wip1^{-/-}p38^{KI/+}* and *wip1^{+/+}p38^{KI/+}* mice during the conditioning training phase (pre- and post-shock, Fig. 5D, $p > 0.05$), and during the tests undertaken the following day in the same context and in a modified environment (Fig. 5E, $p > 0.05$). When we evaluated performance on the T-maze spontaneous alternation task, there was no significant difference between the percentage of spontaneous alternation in *wip1^{-/-}p38^{KI/+}* mice compared with the controls (Fig. 5F, $p > 0.05$).

Together, these data demonstrate that wip1 modulates dendritic spine morphology, associative, contextual and working memory through a p38MAPK-dependent signaling pathway.

Discussion

There is currently a paucity of published information regarding the role(s) of wip1/p38MAPK in the CNS. In the present study, we have revealed novel functions of the wip1/p38MAPK signaling pathway in the CNS. Wip1 phosphatase likely dephosphorylates p38MAPK in the dendritic spines of hippocampal neurons, and can play a modulatory role in dendritic spine morphology, associative and contextual memory.

As reported previously, wip1 mRNA is expressed in the brain¹² and p38MAPK is located in the post synaptic area.¹⁷ Interestingly, in situ analyses in CA1 of the hippocampus of *wip1^{-/-}* mice have revealed a reduction in the dendritic spine head width and length as well as the dendritic spine density. The role of p38MAPK in cytoskeletal organization via its action on the mechanisms of polymerization/depolymerization has been well described.^{17,34} However, we have reported that spine size and density are unchanged in p38MAPK deficient mice. Alteration in the spine morphology caused by p38MAPK deficiency was evident in the *wip1^{-/-}p38^{KI/+}* mice, but was attenuated regarding the dendritic spine density in CA1 of the hippocampus. The reduction in dendritic spine density is higher in wip1 deficient mice (about 70%) than in double mutant mice (about 50%) compared with wild type mice, suggesting wip1 modulates spine morphology through the p38MAPK signaling pathway—but not completely. Interestingly, arcadlin, a protocadherin present in hippocampal neurons, has been shown to act as a “negative” regulator of the number of synaptic spines via p38MAPK and TAO2 β , a MAPK kinase kinase (MAPKKK),³⁵ thus confirming the implied role of p38MAPK in the negative regulation of spine density. In addition, the elimination of wip1 and p38MAPK genes by knockout may also induce a reorganization of the elements involved in regulating the density of the spines in the

hippocampus. In addition other pathways may be involved in this process. It is well established now that once wip1 is induced by p53, wip1 not only directly dephosphorylates p38MAPK (as mentioned above) but Mdm2, p53 and ATM as well. ATM and p-p53 (at serine 15) have been also found expressed in the hippocampus of wip1 deficient mice (compare Fig. S2). Interestingly, mice homozygous for a transgene encoding p44, a short and naturally occurring isoform of the p53, display cognitive decline and synaptic impairment early in life. Recent studies showed that in the absence of p53, wip1 expression can be induced by the JNK-c-Jun pathway, which is activated in Alzheimer disease brain.^{36,37} Activation of the JNK-c-Jun pathway has also been implicated in the decline of hippocampal long-term potentiation.³⁸ Further studies are necessary to identify the molecular mechanisms responsible.

Many phosphatases have been previously involved in memory processes. Acquisition of memory is impaired in calcineurin, PP1 and PP2A deficient mice.^{39,40} In addition, the majority of PP has been found to be downregulated in the brains of Alzheimer disease (AD) sufferers,⁴¹ although activation of calcineurin has also been reported in AD brain.^{42,43} Because AD sufferers display a lack of initiative in addition to anterograde amnesia, it is interesting to examine how closely the mouse model resembles human symptomatology, using the T-maze spontaneous alternation test. Spontaneous alternation has been tested in several murine models of AD, with diverse results. Lower rate of spontaneous alternation have been reported in transgenic mice with the Swedish mutation of the 695 isoform (β APP₆₉₅SWE), or the 770 isoform (β APP₇₇₀SWE) combined with the M146L preselinin mutation compared with non-transgenic mice—and this even before mature A β protein plaques appear.⁴⁴⁻⁴⁶ However, segmental trisomic 16 (Ts65Dn) mice overexpressing β APP and several other genes showed equivalent levels of spontaneous alternation to controls.⁴⁷ Similarly, in our study, the spontaneous alternation rates for mutant wip1 ko mice were statistically comparable to the wild type mice, even if this result was close to the threshold of significance ($p = 0.06$), with an apparent lower (more than 15%) spontaneous alternation level for the mutant mice compared with controls (Fig. 2C). In addition, the results reported in two well established memory tests (contextual fear conditioning and novel object recognition tests) were highly significant for the mutant wip1 mice compared with the wild type ($p = 0.0012$ and $p = 0.0013$, respectively) demonstrating for the first time a role of wip1 in an associative memory task. Interestingly, the disturbance of PP1 anchoring by knockout of the gene encoding spinophilin alters not only learning and memory processes,⁴⁸ but also the formation and the function of

dendritic spines.⁴⁹ Regarding *wip1* heterozygote mice, the dendritic spine was affected in the hippocampus (reduction of 35% compared with controls; Fig. 1B); however, these mice did not show any disturbances of memory in the object recognition test (Fig. 2). Since *wip1* protein is still present in the hippocampus region of the heterozygous mice during the object recognition test, *wip1* can still dephosphorylate its downstream factors (including p38MAPK) and balance the memory deficit. Furthermore, despite experimental findings reporting a role for dendritic spine morphology in mediating learning and memory, the extent of the importance of structural plasticity remains debatable. For instance, it has been estimated that only a small portion of spines formed during training actually contribute to long-term memory.⁵⁰ Thus, even although changes in spine morphology at specific synapses are likely to be important for learning and memory, it is not necessarily the case that all changes in spine morphology will lead to an impairment of memory.

Impairment of associative and contextual memories was observed in the *wip1* deficient mice and reversed in *wip1*^{-/-} *p38*^{KL/+} double mutant mice compared with wild type mice, indicating that *wip1* plays a role in the modulation of these memory processes, at least in part, through the p38MAPK pathway.

Dendritic spine morphology has been correlated with the processes of learning and memory.^{1,5,51} The direction of the causal connection between these events (if any) remains unresolved. Interestingly, alterations of spine density have been previously reported in hippocampus from an AD animal model, β APP₆₉₅SWE transgenic mice.⁵² Substantial morphological changes in dendritic spine density have mainly been observed in the prefrontal cortex and the hippocampus from AD and other dementia sufferers.⁵³ We report that deletion of *wip1* not only leads to abnormal dendritic morphology but also leads to an impairment of learning and memory. The results described here, do not establish any direct causal connection between dendritic spine morphology and memory processes. However, our observations demonstrate novel functions of *wip1* in the CNS in terms of modulating dendrite spine morphology and memory processes, which may provide a better understanding of the molecular mechanism underlying human cognitive disorders. Recent studies of p38MAPK and *wip1* in old mice (22–25 mo old) showed a reduction of *wip1* protein levels during the aging process, which contributes to p38MAPK activation.⁵⁴ Downregulation of *Ppm1d* mRNA expression with aging has also been reported in neural stem cells in the subventricular zone.⁵⁵ Interestingly, *wip1* deficient mice and old (22–25 mo old) wild type mice showed a considerable increase in p38MAPK-dependent signaling, suggesting that *wip1* may play a substantial role in the molecular basis for aging processes through p38MAPK signaling modulation.^{54,56} This finding is consistent with our result that when the expression of *wip1* is decreased or nil there are disturbances in memory. This process seems to be partly p38MAPK-dependent as showed in our results. We have also reported a significant increase in the expression levels of p-p38MAPK in the hippocampus in *wip1* deficient mice measured by immunoblotting (compare Fig. S2A and B). This upregulation of activated p38MAPK was also found when protein fractions (total, nuclear and

cytoplasmic) from the hippocampus of *wip1* deficient mice was analyzed. *Wip1* deficient mice could be a new model of study for aging disorders since the expression of *wip1* is decreased similarly in the hippocampus of the wild type old mice (while p-p38MAPK is increased). In addition, disturbances in memory tasks occurred in *wip1* deficient mice seem to be similar to events occurring during aging). Although much remains to be done, there is promise that a better understanding of the physiological and pathophysiological roles of *wip1* may lead to novel approaches for preventing and treating various disorders, such as aging disorders, neurological pathologies and cancer.

Materials and Methods

Mice. *Wip1*^{-/-} mice (C57BL/6 background) were generated using a targeting construct from a murine genomic *Wip1* clone derived from a 129/Sv phage genomic library as described previously.⁵⁷ *p38*^{KL/+} mutant mice were generated as previously described by targeted mutation of p38MAPK in ES cells.⁵⁴ Briefly, the original BAC clone RP23–83F4 of *Mus musculus* strain C57BL6/J (NCI Intramural Sequencing center) was used to generate targeting vectors. The targeting vectors contained the equivalent of 4.5 kb of mouse DNA with an exon containing the Thr180/Tyr182 sites of p38 α . These sites were subsequently mutated to alanine and phenylalanine by site-directed mutagenesis, leading to an inactivation of phosphorylation of these sites. The entire sequence was placed into the pTK plasmid with two flanking thymidine kinase genes and electroporated into B6 ES cells. The resulting mutated ES cells were used to generate mutant mice.

Wip1^{-/-} *p38*^{KL/+} mice were bred by crossing the *wip1*^{-/-} and *p38*^{KL/+} mice, and their wild type littermates were used as controls. All animal work was conducted under the institutional guidelines of the Animal Ethics Committees of the Singapore General Hospital and the Institute of Molecular and Cellular Biology in Singapore.

Golgi staining. Two- to three-month-old brains of littermate mice were processed and stained according to the protocol provided by the manufacture (FD Neuro Technologies) with some modification. In brief, 100 μ m thick brain sections were cut on a vibratome after impregnation with sodium dichromate buffer. Hippocampus tissue sections were stained with silver nitrate substrate. Reflectance confocal micrographs were obtained using a laser scanning confocal microscope (LSM510 META, Zeiss; 63x objective for dendrite measurements and 40x for the hippocampal neuron images). Each dendrite analyzed was quantified through a z-series stack of 30–60 pictures (depending on the depth of the dendrite) taken at 0.35 to 0.7 μ m intervals. Measurements of dendritic morphology were performed on neurons randomly chosen in the CA1 region of the hippocampus for analysis following previously published criteria.⁵⁸ Briefly, fully impregnated cells were selected and, after identification of the apical shaft, basal dendrites were chosen for analysis. The spine quantification commenced on dendrites starting at more than 85 μ m distal to the soma, and after the first branch point. Measurements were made out to a maximum of 200 μ m from the soma and 20–30 μ m dendritic segments were analyzed (n = 18–27 neurons for each studied genotype) and protrusions were measured

as described previously.⁵⁹ No distinction was made between the different spine types. The analysis of dendritic morphology was performed using Zeiss LSM Image Browser software (Version 4.2) by research assistants who were blind to genotypes and experimental manipulations.

Object recognition test. Memory was assessed by using a modified version of the object recognition test described previously.⁶⁰ The experimental apparatus consisted of a Plexiglas open-field box (55 cm × 41 cm × 35 cm high). The apparatus was located in a sound-attenuated room and was illuminated with a 40 W bulb. The procedure consisted of three sessions: habituation, training, and retention. The animals (3–4 mo old) were videotaped in both training and retention sessions. Each mouse was individually habituated to the box, with 10 min of exploration in the absence of objects for three consecutive days (habituation session, days 1–3). During the training session, two identical objects (A) were placed in two opposite corners of the apparatus 5 cm from the side wall. Each animal was placed in the middle of the apparatus and allowed to explore the objects for 10 min (day 4). An animal was considered to be exploring the object when its head was facing the object (with the distance between the head and object approximately 2 cm or less) or when it was touching or sniffing the object. Ninety minutes after the training session, the animal explored the open field for 10 min in the presence of one familiar (A, the same one used during the training session) and one novel (B) object. Objects were chosen after determining in preliminary experiments that they were equally preferred. Between each trial both the open-field arena and the objects were washed with 70% ethanol solution. All sessions were videotaped, and an experimenter blind to the genotype and experimental conditions analyzed the object recognition behavior. Object placement was counterbalanced so that half of the animals in each treatment group saw the novel object on the left side (relative to the animal's start position) of the open-field arena, and the other half saw the novel object on the right side of the arena. A preference index in the retention session (a ratio of the amount of time spent exploring the novel object over the total time spent exploring both objects) was used to measure cognitive function. In the training session, the recognition index was calculated as a ratio of the time spent exploring the object that was replaced by the novel object in the retention session over the total exploring time.

Contextual fear test. The training chamber was a rectangular box 30 cm × 24 cm × 21 cm in size, equipped with a grid floor composed of stainless steel rods. The grid floor was connected to a shock generator, which delivered a 2 sec, 0.15 mA footshock. The stimulus light and generator were controlled by computer software (TSE Systems). The chamber was cleaned with 70% ethanol between animals. Mice (4 mo old, n = 8–11 per genotype) were trained and tested on two consecutive days. A 2 min pre-training observation period allowed for measurement of basal locomotor activity. Training consisted of placing a subject in the chamber, and allowing exploration for 2 min. Afterwards, an auditory cue (85 dB, Conditioned CS) was presented for 30 sec. The 2 sec footshock (0.15 mA, unconditioned stimulus, US) was administered for the final 2 sec of the CS. This procedure

was repeated, and mice were removed from the chamber 30 sec later.

Twenty four hours after training, mice were returned to the same chamber in which training occurred (context), and freezing behavior was recorded by digital video tracking (Noldus Ethovision). Freezing was defined as lack of movement except that required for respiration. At the end of the 4 min context test, mice were returned to their home cage.

Approximately 2 h later, freezing was recorded in a novel environment context and in response to the cue. The novel environmental context consisted of visual modifications in the chamber, including black bands (3 cm) around the walls of the chamber and plexiglass on the floor. Mice were placed in the novel environment and freezing was recorded for 4 min. The auditory cue (CS) was then presented during the last 2 min. Freezing scores for each subject were expressed as percentage time spent freezing during each portion of the test.

T-maze spontaneous alternation. Performance in the spontaneous alternation task was assessed using a T-maze as previously described.^{61,62} This procedure was performed in an enclosed "T"-shaped maze in which the long arm of the T (40 cm × 10 cm) served as a start arm and the short arms of the T (29 cm × 10 cm) served as the goal arms. Guillotine doors could close the arm entrances from the choice area. Training involved a single forced-choice trial with one goal arm blocked. Testing was for 10 free-choice arm entries or 16 min, whichever occurred first. Mice (4 mo old, n = 8–11 per genotype) placed in the start arm must enter (whole body, including tail) the left or right open goal arm and return to the choice area, and then enter the opposite arm for each trial. At the conclusion of each trial, the maze was cleaned of urine and feces. The number of spontaneous alternations completed (entering the right goal arm after the left goal arm was entered and vice versa), was recorded and expressed as a percentage of total arm entries. To account for differences in the number of entries made, the percentage of alternations accomplished (number of alternating entries achieved/total number of entries completed × 100) was taken as an index of enhanced performance in this task.

Data analysis. Data were analyzed by one way ANOVA, followed by Student's t-test for paired groups (two-tailed) with $p < 0.05$ indicating a significant difference. For the electrophysiological data, ANOVA analysis was performed and followed by Bonferroni corrected post hoc tests, with $p < 0.01$ as a significance threshold.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental materials may be found here:
www.landesbioscience.com/journals/celladhesion/20892

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