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Gender Differences in Spatial Learning, Synaptic Activity, and Long-Term Potentiation in the Hippocampus in Rats: Molecular Mechanisms

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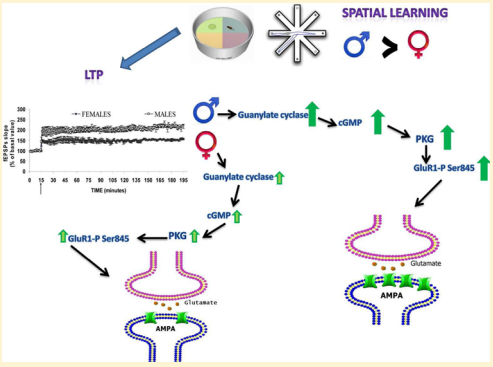
ABSTRACT: In tests of spatial ability, males outperform females both in rats and in humans. The mechanism underlying this gender differential learning ability and memory in spatial tasks remains unknown. Long-term potentiation (LTP) in the hippocampus is considered the basis for spatial learning and memory. The aims of this work were (a) to assess spatial learning and memory in male and female rats in the radial and Morris mazes; (b) to assess whether basal synaptic activity and LTP in the hippocampus are different in male and female rats; and (c) to identify the molecular mechanisms responsible for the gender differences in LTP. We analyzed in young male and female rats (a) performance in spatial tasks in the radial and Morris water mazes; (b) basal synaptic activity in hippocampal slices; and (c) LTP and some mechanisms modulating its magnitude. The results reported allow us to conclude that female rats show larger AMPA receptor-mediate synaptic responses under basal conditions, likely due to enhanced phosphorylation of GluR2 in Ser880 and increased amounts of GluR2-containing AMPA receptors in postsynaptic densities. In contrast, the magnitude of tetanus-induced LTP was lower in females than in males. This is due to reduced activation of soluble guanylate cyclase and the formation of cGMP, leading to lower activation of cGMP-dependent protein kinase and phosphorylation of GluR1 in Ser845, which results in lower insertion of AMPA receptors in the synaptic membrane and a lower magnitude of LTP. These mechanisms may contribute to the reduced performance of females in the radial and Morris water mazes.



KEYWORDS: *AMPA receptor, cGMP, GluR1, cGMP-dependent protein kinase*

Gender differences in performance on various cognitive tasks have been reported. In tests of spatial ability, men outperform women; while in tests of verbal ability, women outperform men.[1,2] In humans, males perform significantly better than females in a virtual Morris water maze,[3,4] indicating better spatial learning and memory. Differences in the acquisition of visual information and in the way this information is applied seem to contribute to the differences in performance. Women look longer at the landmarks than men showing gender dependent cue utilization.[5]

Also in rats males perform better than females in the Morris water maze task.[6−8] The mechanism underlying this gender differential learning ability and memory in spatial tasks remain unknown. Beiko et al.[9] showed sex-differences in water maze performance and suggested that they are due to increased stress during training (with increased serum corticosterone levels) in females. Méndez-López et al.[8] reported a delay in the acquisition of working memory in females. They showed that there are gender-dependent differences in the contribution of limbic structures to the working memory process and suggested that this contributes to the gender differences in spatial working memory.

A relevant difference in spatial learning between male and female rodents seems to be that they employ distinct learning strategies governed by different brain regions. Adult male rats prefer a hippocampus-dependent place strategy over a striatum-dependent response strategy. In contrast, female rats exhibit a preference for a place strategy only when circulating levels of estradiol are elevated. Male rodents typically perform better than females on a variety of spatial learning tasks, which are mediated by the hippocampus.[10]

The hippocampus is the main area involved in the modulation of spatial learning and memory. Animals with hippocampal injuries show impaired perfomance in spatial tasks such as the radial maze test and Morris water maze test.[11−13] Also, humans with damaged hippocampus show impaired performance in the virtual Morris water maze.[13] It is considered that the main mechanism by which the hippocampus modulates spatial learning and memory is long-term potentiation (LTP), an activity-dependent increase in transmission efficacy at synapses.[14−16] Application of tetanus to induce LTP leads to the activation of NMDA receptors, which induces translocation of AMPA receptors to the synaptic membrane that potentiates postsynaptic responses.[17−22] A main mechanism modulating the insertion of GluR1 subunits of AMPA receptors in the synaptic membrane is phosphorylation in Ser845.[23−25] Activation of NMDA receptors increases calcium which activates the

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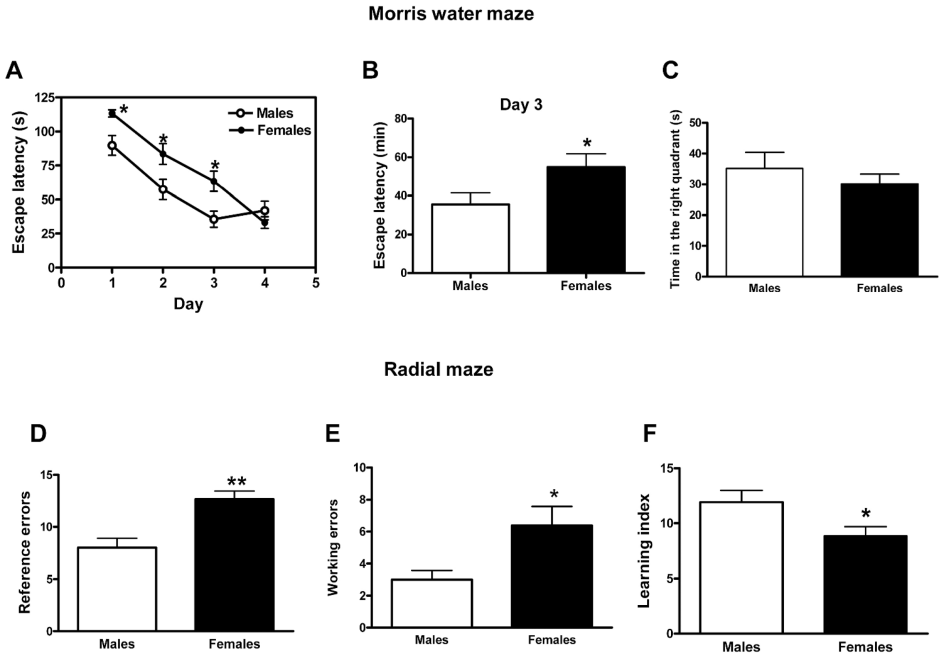

Figure 1. Spatial learning in the Morris water maze and in the radial maze is lower in females than in males. Escape latency in the MWM along the different days is shown in A. Latency on day 3 is shown in B. The time spent in the right quadrant in the test memory on day 5 is shown in C. Values are the mean ± SEM of 18 females and 11 males. In the radial maze, reference errors (D) and working errors (E) are higher and the learning index (F) lower on day 4 of training in females than in males. Values are the mean ± SEM of 18 females and 12 males. Values significantly different between males and females are indicated by asterisks, $*p < 0.05$ and $**p < 0.01$.

glutamate−nitric oxide−cGMP pathway, leading to the activation of cGMP-dependent protein kinase (PKG), which phosphorylates GluR1 in Ser845 and increases the surface expression of AMPA receptors.[26,27] Inhibition of PKG blocks the increase of the AMPA receptor in the membrane and reduces LTP in hippocampal slices.[27]

We hypothesized that gender differences in spatial learning and memory would be associated with differences in hippocampal LTP and in the underlying mechanisms. The aim of the present work was to assess whether differences between male and female rats in spatial learning in the Morris water maze and radial maze are associated with differences in basal synaptic activity and in LTP in the hippocampus and to identify the molecular mechanisms responsible for the gender differences in LTP.

■ RESULTS AND DISCUSSION

Male rats performed significantly better than females both in the Morris water maze (MWM) and in the radial maze tests (Figure 1). As shown in Figure 1A, the latency to find the platform was longer in females than in males at days 1, 2, and 3. For example, on day 3, the escape latency was longer (59 ± 7 s, $p < 0.05$ $Df = 28$, $t = 2.1$, and $F(15, 13) = 1.57$ in females than in males (38 ± 7 s) (Figure 1B). After the 4 training days, spatial memory was assessed 24 h later (on the fifth day) by removing the platform and assessing the time spent in the right quadrant. As shown in Figure 1C, no difference was found between males and females in this memory test. We believe that this is due to the fact that, as shown in Figure 1A, at day 4 both males and females have already learned the spatial position, and no differences are found thereafter.

In the radial maze, on day 4, female rats made more ($p < 0.01$, $Df = 34$, $t = 3.91$, $F(20, 14) = 1.00$ reference (12.4 ± 0.9) (Figure 1D) and working (6.2 ± 1.2, $p = 0.014$ $Df = 31$, $t = 2.6$, $F(15, 16) = 4.00$ errors (Figure 1E) than males (8 ± 1 and 3.1 ± 0.7, respectively). The learning index was also significantly ($p < 0.05$, $Df = 24$, $t = 2.3$ $F(11, 13) = 1.43$ lower (8.9 ± 0.8) in females than in males (11.9 ± 1.1) (Figure 1F).

We then analyzed whether synaptic activity in the CA1 region of the hippocampus is different in males and females. As shown in Figure 2A, under basal conditions, the synaptic responses evoked by stimuli of different intensities were lower in males than females. The magnitude of the basal excitatory postsynaptic potentials (EPSPs) is significantly lower ($p < 0.0001-0.05$, $Df = 1$, $F = 280$) in slices from males for stimuli in the range between 120 and 500 μA (Figure 2A).

We assessed the contribution of AMPA or NMDA to the lower response in males by measuring the amplitude of the EPSPs evoked by activation of these receptors. The amplitude of the AMPA receptor-mediated EPSP was around 60% lower in males ($p < 0.0001-0.05$ $Df = 1$, $F = 190$) for any stimulation intensity (Figure 2B). NMDA receptor-mediated-EPSPs are not significantly different in males and females (Figure 2C). These results show gender differences in AMPA receptor-mediated neurotransmission in the hippocampus under basal conditions.

We assessed whether the higher AMPA receptor-mediated EPSP in females is due to an increased amount of these receptors. Under basal conditions, the content of the GluR1 subunit of the AMPA receptor was similar in males and females, both in the whole homogenate and in the fraction rich in postsynaptic densities (PSD fraction) (Figure 3A). However, as shown in Figure 3B, the amount of the GluR2 subunit in the PSD fraction was higher in females (170 ± 30% of males, $p < 0.05$, $Df = 14$, $t = 2.2$, $F(7, 7) = 9.00$. Moreover, the phosphorylation of this subunit in Ser880 was also higher in females (145 ± 14% of males, $p < 0.005$ $Df = 14$, $t = 2.9$, $F(7, 7) = 9.00$ (Figure 3B).

The content of alpha 1, alpha 2, and gamma 2 subunits of $GABA_A$ receptors was similar in males and females. The contents

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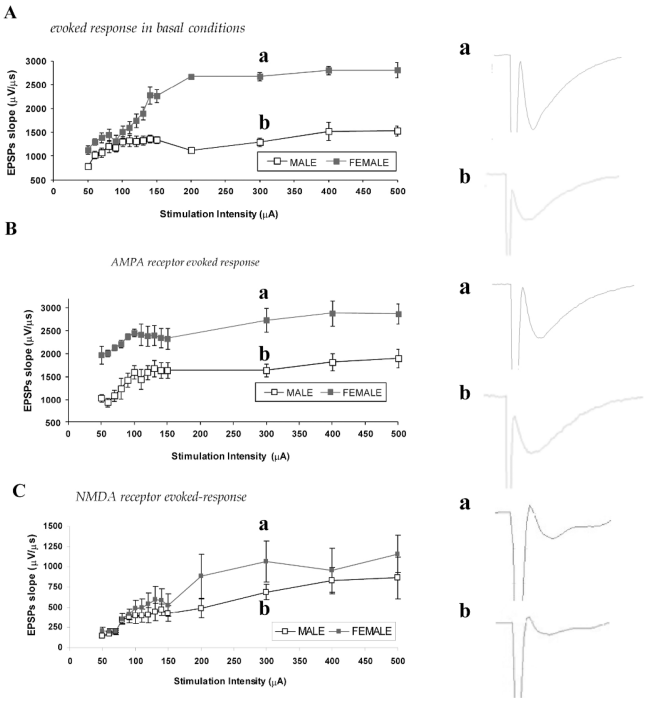

Figure 2. Total evoked responses and AMPA and GABA-receptor-mediated responses are higher in female than male rats under basal conditions. Stimuli of different intensities were applied to hippocampal slices from male (white squares) or female (black squares) rats. The slope of the EPSPs mediated in basal conditions is shown in A, those mediated by AMPA receptors in B, and by NMDA receptors in C. Values are the mean ± SEM of 8 rats per group. In A, values from 120 to 500 μA for male rats were significantly lower than those for females rats ($p < 0.0001-0.05$). In B, all values for males rats were significantly lower than those for females rats ($p < 0.0001-0.05$). In C, values were not significantly different between males and females. Typical raw EPSPs registered at 300 μA (as an example) are shown for female (a) and male (b) rats.

in females, expressed as the percentage of value for males were alpha 1, 105 ± 4%; alpha 2, 99 ± 5%; and gamma 2, 103 ± 12%.

We then assessed the effects of application of tetanus to induce LTP on the magnitude of EPSPs. As shown in Figure 4, the

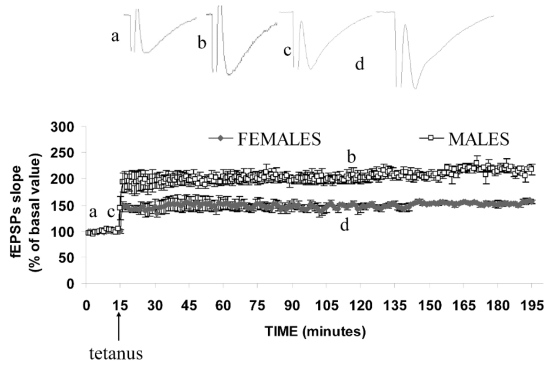

Figure 4. Magnitude of the LTP in the CA1 area of the hippocampus is lower in female than in male rats. Electrophysiological experiments were carried out as described in the Methods section with hippocampal slices from male or female control rats. Schaffer-collateral-commissural pathways were stimulated with electrical pulses, and at the time indicated by the arrow, tetanus was applied. White and black squares show the fEPSP amplitude in slices from males and females, respectively (mean ± SEM, $n = 6$). (A) Typical raw EPSPs at the indicated times are shown for male rats (a,b) and for female rats (c,d). (B) Both in male and female rats, the fEPSP slope after tetanus was significantly different from the basal values (before application of the tetanus; $p < 0.0001$ and $p < 0.001$, respectively). The magnitude of LTP was significantly lower (159.5 ± 3.8) in females than in males (225 ± 6.8) ($p < 0.05$).

magnitude of LTP in CA1 was lower in females than in males. The fEPSPs slope at 3 h after tetanus was 225 ± 7% of basal values for males and 159 ± 4% for females ($p < 0.0001$, $Df = 10$, $t = 8.4$ $F(5, 5) = 3.20$ (Figure 4).

As the magnitude of LTP is mainly modulated by the insertion of AMPA receptors in the synaptic membrane, we assessed

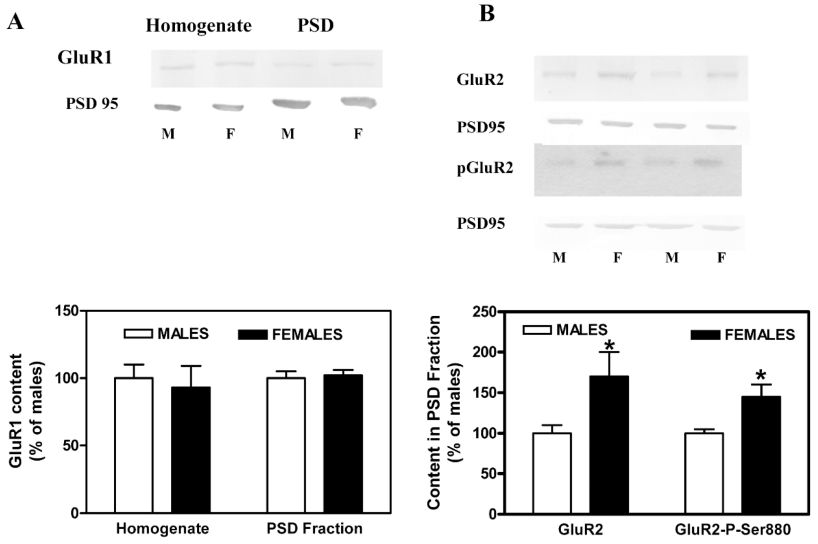

Figure 3. Gender does not affect the amount of GluR1 in total homogenate or PSD, but the content of GluR2 and its phosphorylation in Ser880 are higher in PSD in females. Whole homogenates and PSD from hippocampi of males or females rats were subjected to immunoblotting to analyze the amount of GluR1 (A) and GluR2 subunits and phosphorylation of GluR2 in Ser880 (B). Typical immunoblots are shown above each Figure. The intensities of the bands were quantified and expressed as percentage of male rats. Values are the mean ± SEM of eight rats per group. Values significantly different in male and female rats are indicated by asterisks *$p < 0.05$.

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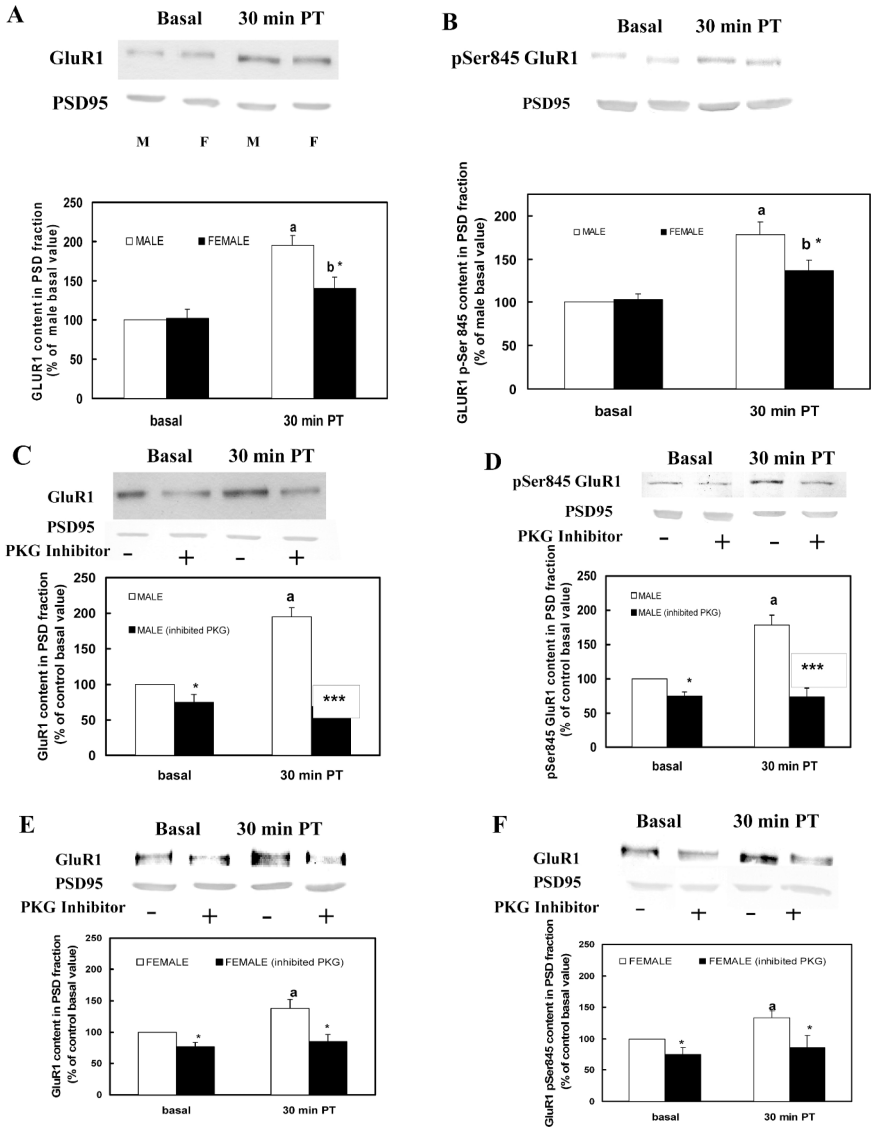

Figure 5. Tetanus-induced phosphorylation of GluR1 in Ser845 and recruitment of GluR1 to synaptic membranes are lower in females than in males and are prevented by the inhibition of PKG. PSD fractions were prepared from hippocampi of males or females rats under basal conditions or 30 min after tetanus application. The content of GluR1 (A) and its phosphorylation in Ser845 (B) was analyzed by immunoblotting. Similar experiments were performed in the presence of an inhibitor of PKG in males (C,D) and females (E,F). The intensities of the bands were quantified, and the amount and phosphorylation of GluR1 are expressed as a percentage of male rats under basal conditions. Typical immunoblottings are shown above each Figure. Values are the mean ± SEM of 6 rats per group. Values significantly different from basal ones (before tetanus application) are indicated by "a" for male rats ($p < 0.005$) and by "b" for females rats ($p < 0.05$). Values significantly different in males rats than in females rats are indicated by asterisks *$p < 0.05$.

whether tetanus-induced translocation of AMPA receptors is different in males and females. Although the content of the GluR1 subunit in PSD under basal conditions is similar in males and females, the response to tetanus is different. The tetanus-induced increase in GluR1 content in PSD (Figure 5A) is significantly ($p < 0.005$, $Df = 10$, $t = 2.98$, $F(5, 5) = 1.16$ lower in females (138 ± 14% of basal) than in males (195 ± 13% of basal).

As tetanus-induced translocation of GluR1 to the synaptic membrane is mediated by its phosphorylation in Ser845, we analyzed the phosphorylation of GluR1 in Ser845 (Figure 5B). In slices from males, tetanus increases phosphorylation to 178 ± 14% of basal values ($p < 0.001$ $Df = 10$, $t = 5.3$, $F(5, 5) = 7.84$. The increase was significantly lower in females (137 ± 12% of basal, $p < 0.05$, $Df = 10$, $t = 2.2$, $F(5, 5) = 1.36$.

To assess the role of PKG in tetanus-induced phosphorylation and translocation of GluR1, we repeated the experiment in the presence of an inhibitor of PKG. Rp-8-pCPT-cGMPS (8- (4-Chlorophenylthio)guanosine-3′,5′-cyclic monophosphorothioate), a membrane-permeant and phosphodiesterase-resistant PKG inhibitor (Biolog Cat. No C 013−01) was perfused at 10 μM starting 30 min before tetanus.

In males, inhibition of PKG reduces ($p < 0.05$, $Df = 10$ $t = 2.3$ $F(5, 5) = 30.25$ the content of GluR1 in PSD to 74 ± 11% of basal values (Figure 5C) and its phosphorylation to 75 ± 6% ($p < 0.05$, $Df = 10$, $t = 3.9$, $F(5, 5) = 9.00$ under basal conditions (Figure 5D). Inhibition of PKG completely abolishes ($p < 0.0001$, $Df = 10$, $t = 6.3$, $F(5, 5) = 1.69$ compared with slices without PKG inhibitor) tetanus-induced increase of GluR1 content in PSD, which remains at 92 ± 10% of basal values (Figure 5C) and tetanus-induced phosphorylation, which remains at 98 ± 13% of basal values (Figure 5D) ($p = 0.0019$, $Df = 10$, $t = 4.2$, $F(5, 5) = 1.16$ compared with a slice without the PKG inhibitor).

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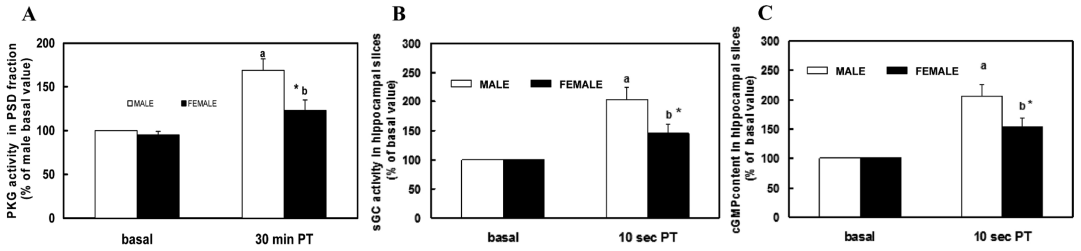

Figure 6. Tetanus-induced activation of PKG and of guanylate cyclase, and formation of cGMP are lower in female rats. Hippocampal slices from male and female rats were subjected or not to the tetanic stimulation as indicated. All samples (with or without tetanus) were taken at the same time, 30 min after tetanus administration. The activity of PKG (A) and guanylate cyclase (B) and the content of cGMP (C) were measured as indicated in Methods. Values are the mean ± SEM of 6 rats per group and are expressed as percentage of values in slices from males under basal conditions. Values which are significantly increased by the tetanus compared with their basal content are indicated by "a" for male ($p < 0.001$) and by "b" for females ($p < 0.05$). Values that are significantly different (at the same time) in males and females are indicated by asterisks: *$p < 0.05$.

Similar results were obtained in females. Inhibition of PKG reduces ($p < 0.05$, Df =10, t = 1.8, $F(5, 5)$ = 2.47 the content of GluR1 in PSD to 77 ± 7% of basal values (Figure 5E) and its phosphorylation to 76 ± 10% ($p < 0.05$, Df = 10, t = 2.3, $F(5, 5)$ = 6.25 under basal conditions (Figure 5F). Inhibition of PKG completely abolishes tetanus-induced increase of GluR1 content in PSD, which remains at 111 ± 11% of basal conditions (Figure 5E) ($p = 0.16$, Df = 10, t = 1.5, $F(5, 5)$ = 1.62 compared with slices without PKG inhibitor) and tetanus-induced phosphorylation, which remains at 115 ± 19% of basal ($p = 0.35$, Df = 10, t = 1.0, $F(5, 5)$ = 2.51 compared with slices without the PKG inhibitor) (Figure 5F).

As activation of PKG mediates tetanus-induced insertion of GluR1 in PSD, we assessed if PKG activation is different in males and females. As shown in Figure 6A, basal activity of PKG was not different in males and females. However, application of tetanus increases PKG activity in PSD significantly more (169 ± 13% of basal, $p < 0.001$, Df = 10, t = 5.6, $F(5, 5)$ = 5.30 in males than in females (129 ± 12% of basal, $p < 0.05$, Df = 10, t = 2.3, $F(5, 5)$ = 3.92 30 min after tetanus (*$p = 0.014$, Df = 10, t = 3.0, $F(5, 5)$ = 1.35.

As tetanus-induced activation of PKG is due to the activation of guanylate cyclase and increased cGMP levels,[26,28] we assessed whether these are different in males and females. As shown in Figure 6B, tetanus-induced activation of guanylate cyclase is lower ($p = 0.076$, Df = 10, t = 2.0, $F(5, 5)$ = 1.68 in females (145 ± 16% of basal, $p < 0.05$, Df =10, t = 2.8, $F(5, 5)$ = 11.56 than in males (204 ± 20% of basal, $p < 0.005$, Df = 10, t = 5.7, $F(5, 5)$ = 19.36. Also, the tetanus-induced increase in cGMP content is lower ($p = 0.091$, Df = 10, t = 1.9, $F(5, 5)$ = 1.68 in females (154 ± 15% of basal, $p < 0.01$, Df =10, t = 3.2, $F(5, 5)$ = 11.56 than in males (206 ± 19% of basal, $p < 0.0005$, Df =10, t = 4.8, $F(5, 5)$ = 19.36 (Figure 6C).

The results reported show that there are important gender differences in spatial learning and in basal synaptic activity and long-term potentiation in the hippocampus. Female rats show larger synaptic responses under basal conditions, mainly due to enhanced neurotransmission mediated by AMPA receptors, likely due to enhanced phosphorylation of GluR2 in Ser880 and a larger amount of GluR2-containing AMPA receptors in postsynaptic densities.

In contrast, the magnitude of LTP in response to tetanus is significantly lower in females than in males. This is due to reduced activation of soluble guanylate cyclase and the formation of cGMP, leading to lower activation of PKG and phosphorylation of GluR1 in Ser845, which results in lower insertion of AMPA receptors in the synaptic membrane and a lower magnitude of LTP. The mechanisms leading to this reduced LTP would be also involved in the reduced ability to learn the spatial tasks in the radial and Morris water mazes.

The GluR2 subunit controls calcium permeability of AMPA receptors and plays a critical role in controlling the assembly and stability of AMPA receptors.[29,30] The larger AMPA synaptic responses under basal conditions in female rats would be due to the larger amount of GluR2-containing AMPA receptors in the PSD. This would be due to the increased phosphorylation in Ser880, which plays an important role in the modulation of trafficking of GluR2-containing AMPA receptors to the synaptic membrane.[30,31] Phosphorylation of GluR2 is compartmentally restricted to receptors located at the cell surface, and Ser880-phosphorylation retains AMPA receptors in the synaptic membrane.[32] The gender differences in the phosphorylation of GluR2 would explain the differences in the content of GluR2 in the PSD fraction and the larger AMPA response in females under basal conditions.

We previously reported that the magnitude of LTP in the CA1 region of the hippocampus is significantly lower in young female than in male rats.[33] We now provide new insights on the mechanisms leading to lower LTP. The magnitude of LTP depends on the amount of AMPA receptors inserted in the synaptic membrane, which, in turn, is modulated by the phosphorylation of GluR1 in Ser845.[19,20,23,25,34]

Liu et al.[34] showed that an aptamer A2 can effectively abrogate the phosphorylation of GluR1 at Ser845 and inhibit GluR1/GluR1-containing AMPA receptor trafficking to the cell surface. Oh et al.[25] showed that changes in Ser-845 phosphorylation were paralleled by corresponding changes in the surface expression of AMPARs in both cultured neurons and hippocampal slices. Phosphorylation of GluR1 in Ser845 is critical for proper induction of LTP.[35] Mice lacking both Ser845 and Ser831 phosphorylation sites show reduced and faster decaying LTP.[24]

These studies clearly show that phosphorylation of GluR1 in Ser845 modulates membrane expression of AMPA receptors and the magnitude of LTP. This supports the fact that the reduced magnitude of LTP in females is due to the reduced tetanus-induced increase of AMPA receptors in synaptic membranes, which in turn is due to the reduced phosphorylation of GluR1 in Ser845.

This reduced phosphorylation of GluR1 in Ser845 would be due to the lower activation of PKG in females. GluR1 is phosphorylated at different sites by different protein kinases.[36,27,24,37,35] Moreover, GluR1 forms a complex with PKG. Activation of PKG in this complex by cGMP results in the phosphorylation of GluR1 in Ser845 and increased surface

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expression of AMPA receptors.[27,35] Moreover, inhibition of PKG blocks the increase of AMPA receptors in the membrane[27,35] and reduces LTP in hippocampal slices.[38] Therefore, the lower activation of PKG in females would be responsible for the lower LTP.

Reduced tetanus-induced activation of guanylate cyclase and cGMP increase would be responsible for the lower activation of PKG in females. Guanylate cyclase is activated following tetanus-induced activation of NMDA receptors, which increases calcium in the postsynaptic neuron, leading to activation of nitric oxide synthase and formation of nitric oxide, which activates guanylate cyclase.[26] The lower increase in guanylate cyclase may be due to the reduced amount of NMDA receptors in the synaptic membrane in females compared to males.[33]

The lower magnitude of LTP in females would contribute to their lower ability in spatial learning and memory. The role in spatial learning and memory of hippocampal NMDA-receptor-dependent LTP is supported by reports showing that blocking NMDA receptors impairs both LTP and spatial learning.[39,40] Also, mice lacking spine apparatuses (synaptopodin-deficient mice) or the GluR-A subunit show deficits in LTP and impaired spatial learning and working memory.[41−43]

In summary, we show that female rats show enhanced AMPA receptor-mediated synaptic activity under basal conditions but a lower potentiation of AMPA responses in response to tetanus. The lower magnitude of LTP in females is due to a lower increase of cGMP- and PKG-mediated phosphorylation of GluR1 in Ser845, resulting in reduced increase of insertion of AMPA receptors in the synaptic membrane and lower magnitude of LTP, which, in turn, would contribute to the reduced performance of females in spatial learning and memory tasks.

METHODS

Animals. Female and male Wistar rats (2−3 months-old) were used. Adequate measures were taken to minimize pain and discomfort to the animals. The experiments were approved by the Comite de Experimentación y Bienestar Animal (CEBA) of our Center and were performed in accordance with guidelines of the Directive of the European Commission (2010/63/EU) and Spanish legislation (R.D. 1201/2005) for the care and management of experimental animals.

Spatial Learning in the Morris Water Maze. The test was carried out as described by Monfort et al.[44] using a circular pool (160 cm diameter, 40 cm height) arbitrarily divided into four quadrants. After pretraining, the rats were trained to learn the fixed location of the invisible platform during 4 days. Each training trial involved placing the rat into the pool facing the wall at one of the three quadrants lacking the platform. A different starting point was randomly used on each trial. Training consisted of three swims per day. Each animal was allowed a maximum of 120 s to find the platform and was left for 20 s on the platform. If a rat failed to locate the platform within 120 s, it was then manually guided to the platform by the experimenter. The time needed to find the hidden platform was recorded manually and used as a measure of learning of the task. To assess spatial memory, the platform was removed from the pool and 24 h after the last training trial the rats were placed again in the pool for 60 s, and the time spent in the quadrant where the platform was located was recorded.

Spatial Learning in the Radial Maze. The apparatus is composed of a central area that gave access to eight equally sized arms. The arms were 70 cm long and 10 cm wide, and the central area was 30 cm in diameter. The distal extreme of each arm had a cup for the food rewards. Rats were allowed to explore the maze for 10 min on two consecutive days in the presence of distal cues (posters and objects of different sizes), which remained in place throughout training. Training in the radial maze was composed of five blocks of three trials each, performed on 10 consecutive days. The task involved locating four pellets, each placed at the end of a different arm according to a random configuration as described by Hernandez-Rabaza et al.[45] The number of spatial reference errors (reference memory errors, first visits to unbaited arms) and working memory errors (working errors, visits to arms already visited in the same trial) were calculated and expressed as the number of reference and working errors per block. A learning index was calculated as the number of right choices minus the number of errors in the first entry into each arm.

Long-Term Potentiation in Hippocampal Slices. Experiments were performed using transverse hippocampal slices (400 μm) as previously described.[47] Schaffer collateral-comissural fibers were stimulated with electrical square pulses of 50−300 μA range, 40 μs, 0.05 Hz (Grass S88 stimulator) applied through bipolar tungsten microelectrodes located in a set of the fibers in the stratum radiatum. Evoked field potentials were recorded from CA1 stratum radiatum with low resistance glass micropipettes filled with Ringer solution. Recording micropipettes were connected to field effect transistors, the outputs were filtered between 1 Hz and 3 kHz and amplified by CyberAmp 380 (Axon Instruments). Evoked responses were online digitized at 10 kHz (Digidata 1200 Interface, Axon Instruments). Once the evoked potentials were stable, basal potentials were recorded for 15 min. Then, a high frequency stimulation (HFS) consisting of a tetanus of 3 trains (100 Hz, 1s) at 20 s intervals was given to induce long-term potentiation (LTP). The synaptic strength was calculated by measuring the slope of the field EPSP (fEPSP). EPSPs after tetanus were normalized by referring them to the mean values of responses during the initial 15 min period, before tetanus application.

Recording of Evoked Postsynaptic Potentials Due to the Activation of AMPA or NMDA Receptors. Experiments were performed using transverse hippocampal slices (400 μm) as described above. To avoid epileptic discharges, the CA3 region of the slices was cut off. Schaffer collateral-comissural fibers were stimulated with electrical pulses of 100−400 μA, 40 μs, and 0.05 Hz applied through bipolar tungsten microelectrodes located in the stratum radiatum, and the field excitatory postsynaptic potentials (fEPSPs) in the CA1 were recorded as described above. Once the basal evoked potentials were stable, slices were incubated with KRB containing 50 μM picrotoxin to inhibit the response-mediated by GABA receptors. To evaluate the function of AMPA receptors, we measured the magnitude of the EPSPs evoked by application of electrical stimulus with different intensities in the presence or absence of 20 μM CNQX, an AMPA receptor antagonist. The AMPA receptor evoked response is calculated as the difference of the magnitude of the EPSPs in the absence and presence of CNQX. To evaluate the function of NMDA receptors, we used a medium with low magnesium concentration and blocked AMPA receptors with 20 μM CNQX. The magnitude of the EPSPs evoked by application of electrical stimulus with different intensities in the presence or absence of 100 μM of AP5, an NMDA receptor antagonist, was measured. The NMDA receptor evoked response is calculated as the difference of the magnitude of the EPSPs in the absence and presence of AP-5.

The experiments were performed by recording first the AMPA receptor-mediated component of the EPSP as described above. Then, the same slices were perfused with KRB containing 0.1 instead of 1.3 mM $MgSO_4$, 50 μM picrotoxin, and 20 μM CNQX, and the NMDA receptor-mediated postsynaptic potentials were registered as described above. To confirm that the potentials measured are mediated by activation of NMDA receptors, 100 μM AP5 was perfused after every experiment to block NMDA receptors and associated EPSPs.

Preparation of a Fraction Rich in Postsynaptic Density (PSD). We obtained triton-insoluble fraction (TIF) according to Picconi et al.[46] The hippocampal slices were rapidly homogenized in cold 0.32 M sucrose containing (in mM) 1 HEPES, 1 $MgCl_2$, 1 $NaHCO_3$, and 0.1 PMSF, pH 7.4, in the presence of a complete set of protease inhibitors. The homogenized slices were centrifuged at 1000*g* for 10 min. The resulting supernatant was centrifuged at 3000*g* for 15 min to obtain a fraction of mitochondria and synaptosomes. The pellet was resuspended in hypotonic buffer in a glass potter and centrifuged at 100,000*g* for 1 h. The pellet was resuspended in 100 μL of buffer containing 75 mM KCl and 1% Triton X-100 and centrifuged at 100,000*g* for 1 h. The final pellet was homogenized in a glass−glass potter in 20 mM HEPES in the presence of a complete set of protease inhibitors. An equal volume of

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glycerol was added, and samples were stored at −80 °C. This fraction is referred to as the "Triton insoluble fraction" (TIF). The TIF was used instead of the classical PSD preparation because the amount of the starting material was very limited.

However, the protein composition of this preparation was carefully tested for the absence of presynaptic markers (i.e., synaptophysin and synaptotagmin) and for the enrichment in the PSD proteins (CaMKII, PSD-95, NMDA, and AMPA receptor subunits).[48]

Immunoblotting. Whole homogenates or PSD fractions from slices obtained as above were subjected to immunoblotting as described in ref 49 using antibodies against the GluR1 subunit of the AMPA receptor (1:100; Calbiochem Germany), GluR1 phosphorylated in Ser845 (1:600; Abcam, UK), GluR2, GluR2 phosphorylated in Ser880, alpha 1, alpha 2, and gamma 2 (GABA receptor subunits), and PSD95 (1:1000; Calbiochem, Germany). After development using antirabbit IgG conjugated with alkaline phosphatase (Sigma, Germany) and alkaline phosphatase color development (Sigma), images were captured, and the intensities of the bands were measured using the AlphaImager 2200 program.

Determination of Soluble Guanylate Cyclase Activity and cGMP Content in Hippocampal Slices. This was performed essentially as in Monfort et al.[26] Hippocampal slices were collected under basal conditions (without tetanus) and at 10 s after the application of tetanus. Slices were homogenized in ice-cold buffer containing 50 mM HEPES, pH 7.4, 4 mM EDTA, 0.01% bacitracin, 50% glycerol, 250 mM sucrose, and 1 mM dithiothreitol. The homogenates were centrifuged for 45 min at 430,000*g* (4 °C). Fifty microliters of the supernatant was used to measure cGMP concentration using the BIOTRAK cGMP enzymeimmunoassay kit from Amersham and another 50 μL to measure soluble guanylate cyclase activity.

Determination of Protein Kinase G (PKG) Activity in the PSD Fraction. Hippocampal slices were collected under basal conditions (without tetanus) and at 30 min after application of the tetanus. Slices were homogenized in ice-cold buffer containing 50 mM Tris-HCl, pH 7.4, 0.1 mg/mL BSA, 5 mM mercaptoethanol, 20 mM magnesium acetate, 0.01% Triton X-100, 10 μg/mL leupeptine, 10 μg/mL aprotin, 0.2 mM IBMX, 1 mM $NaVO_4$, 1 mM NaF, 2.5 mM dithiothreitol, and 20 μM PKI (cAMP-dependent protein kinase inhibitor). From these homogenates, fractions rich in PSD were obtained (as above) and used to measure the PKG activity using a non-radioisotopic kit (Cyclex cyclic GMP-dependent protein kinase assay kit, MBL, Woburn, MA, USA). This cGK assay kit is based on the use of a peroxidase coupled phospho-specific monoclonal antibody that recognizes the phospho-threonine 68/119 residues on a substrate phosphorylated by PKG.

Statistical Analysis. The data are the mean ± SEM of the number of experiments indicated in the legend to each figure. Statistical analysis was carried out using ANOVA followed by Tukey's posthoc test. When only two values were compared, Student's *t* test was used. A value of $p < 0.05$ was considered significant.

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Author Contributions

P.M. performed all the in vitro experiments in hippocampus (LTP, protein content and enzyme activities) and contributed to analysis of the results and preparation of the figures. B.G.-G. performed behavioral experiments: Morris water maze and Radial maze. M.L. contributed to analysis of the results and preparation of the figures. V.F. contributed to analysis of the results and has written the manuscript.

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Notes

The authors declare no competing financial interest.

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