Published in final edited form as:

FASEB J. 2020 January ; 34(1): 706–719. doi:10.1096/fj.201902217R.

Rapid actions of anti-Müllerian hormone in regulating synaptic transmission and long-term synaptic plasticity in the hippocampus

Kang Wang*, Fuhua Xu*, Shawn P. Campbell*, Kyle D. Hart*, Tyler Durham[†], James Maylie*,
¹, Jing Xu*,^{†,2}

*School of Medicine, Oregon Health & Science University, Portland, Oregon, USA

[†]Oregon National Primate Research Center, Oregon Health & Science University, Beaverton, Oregon, USA

Abstract

Anti-Müllerian hormone (Amh) is a peptide factor that is known to regulate sexual differentiation and gonadal function in mammals. Although Amh is also suggested to be associated with cognitive development and function in the postnatal brain, little is known about its expression or direct effects on neuronal activities in the hippocampus. Therefore, we assessed Amh and its receptor expression in the hippocampus of male and female mice using PCR, Western blot, and immunofluorescence staining. While Amh-specific receptor expression was comparable between males and females, mRNA and protein levels of Amh were higher in females than those of males. Electrophysiological recordings on acute hippocampal slices showed that exogenous Amh protein addition increased synaptic transmission and long-term synaptic plasticity at the Cornu Ammonis (CA) 3-CA1 synapses. Amh exposure also increased the excitatory postsynaptic potential at CA1 synapses. Our findings support direct and rapid actions of Amh as a paracrine and/or autocrine factor in regulating hippocampal neuronal activities. Data provide functional evidence of Amh-mediated postsynaptic modulation of synaptic transmission and Amh-regulated long-term synaptic plasticity in the hippocampus. These results suggest a potential role of Amh in learning and memory, and a possible cause of the sex differences in cognitive development and function.

Keywords

anti-Müllerian hormone receptor 2; postsynaptic modulation; cognitive development; cognitive function; electrophysiological recording

¹Correspondence: Oregon Health & Science University, 3181 SW Sam Jackson Park Road, Portland, Oregon 97239, USA. mayliej@ohsu.edu. Telephone: +1-503-494-2106. Fax: +1-503-494-5296. ²Correspondence: Oregon Health & Science University, 505 NW 185th Avenue, Beaverton, Oregon 97006, USA. xujin@ohsu.edu. Telephone: +1-503-346-5411. Fax: +1-503-346-5585.

AUTHOR CONTRIBUTIONS

K. Wang, J. Maylie, and J. Xu designed research; K. D. Hart, J. Maylie, and J. Xu analyzed data; K. Wang, F. Xu, S. P. Campbell, T. Durham performed research; K. Wang, J. Maylie, and J. Xu wrote the paper.

Introduction

Anti-Müllerian hormone (Amh), also known as Müllerian-inhibiting substance, is a glycoprotein that was originally recognized as a fetal hormone generated by the testicular Sertoli cells to regulate sexual differentiation in mammalian species (1). Throughout the lifespan, the testis continuously secretes Amh which acts as a paracrine factor in supporting testicular development and function to maintain male physical characteristics. In females, granulosa cells of the postnatal ovary begin to produce Amh upon the initiation of follicular development to support ovarian steroidogenic and gametogenic function (2–4). Recently, Amh has also emerged as a neuroactive peptide in regulating neuronal viability and activity in various brain regions (5, 6).

As a ligand of the transforming growth factor beta family (TGF- β), Amh activates downstream signaling by binding to a specific type II transmembrane serine/threonine kinase receptor (Amhr2) which subsequently recruits type I receptors of bone morphogenetic proteins encoded by activin receptor-like kinase 2/3/6 (*Alk2/3/6*) (7). Data on Amhr2 expression in the human brain are limited, except one study showing Amhr2 immunoreactivity in neurons of the hypothalamus (6). Nevertheless, Amhr2 protein expression is identified in most of the brain regions in mice during adulthood (8), including the hippocampus (5, 6) which is a component of the limbic system located under the cerebral cortex. The evidence suggests the potential for direct actions of Amh in regulating hippocampal functions. However, functional studies have not been performed in any animal models.

The hippocampus plays important roles in the consolidation of information from short-term memory to long-term memory, in spatial memory and navigation, as well as in the cognitive process of environmental stimuli (9). The neural circuit includes two major subfields, i.e., Cornu Ammonis (CA) 1 and CA3 containing pyramidal neurons. Although null mutation of *Amh* or *Amhr2* is very rare in humans, which limits detailed studies of the potential role of Amh in hippocampal function in patients, a population-based cohort study demonstrated a significant association between *Amh* single nucleotide polymorphisms and cognitive function parameters in man (10). Clinical studies also indicated the possible involvement of Amh actions in male bias in autism (11), in protection of the hippocampal neurons in women with epilepsy (12), and in the sex differences in cognitive development of children (13). However, the source of Amh ligand in the brain requires a thorough investigation. The known sources of circulating Amh in adult mammals are gonads, i.e., the testis and the ovary (2, 4). A recent study in mice demonstrated that Amh in the circulation did not cross the blood-brain barrier (14), a highly selective barrier operating at birth.

Therefore, experiments were designed using adult mice to test the hypotheses that (a) Amh is present in the cerebrospinal fluid (CSF) (15, 16); (b) Amh and its receptors, especially the ligand-specific Amhr2, are expressed in the hippocampus; and (c) Amh regulates hippocampal synaptic transmission via direct actions on hippocampal CA1 neurons by altering CA3-CA1 excitatory synaptic transmission and long-term synaptic plasticity.

MATERIALS AND METHODS

Animals

All animal protocols were approved by the Institutional Animal Care and Use Committee in the Oregon Health & Science University (OHSU). Animals were treated according to the National Institutes of Health guidelines. Wild-type adult male and female CD-1 mice (7–12 weeks old) were purchased from the OHSU Transgenic Mouse Model Core, and were group housed (3–5 per cage) in a temperature- and light-controlled environment in the Division of Comparative Medicine, OHSU. Food and water were provided ad libitum.

Amh levels in the blood and in the CSF

To measure Amh levels in the blood, trunk blood was collected following decapitation of male ($n = 5$) and female ($n = 5$) mice. Plasma was analyzed for Amh protein concentrations using an AMH (Rat and Mouse) ELISA kit (AnshLabs, Webster, TX, USA) by the Endocrine Technologies Core in the Oregon National Primate Research Center (ONPRC) at OHSU based on the manufacturers' instructions. The standard curve of the assay ranged 0.23–15 ng/ml.

Because only 10–15 μ l of CSF could be collected without blood contamination from individual mouse (17), Western blot was used to evaluate Amh levels in the CSF, as previously described (18). Briefly, CSF samples from male ($n = 3$) and female ($n = 3$) mice (BioIVT, Westbury, NY, USA) were electrophoresed on 4–12% Bis-Tris gel and transferred to a nitrocellulose membrane. The membrane was blocked using 5% nonfat milk in 10 mM Tris-buffered saline (pH 8.0) and then incubated with primary antibodies anti-mouse Amh (1:1000; MAB1737; R&D Systems, Minneapolis, MN, USA) and albumin (loading control; 1:500; MAB1455; R&D Systems) at 4 °C overnight. The specificity of Amh antibody was characterized by immunohistochemistry using ovarian sections in previous studies (20). Positive staining was only detected in granulosa cells of growing follicles expressing Amh. Antibody preabsorbed with blocking peptide was used as a negative control. The membrane was subsequently incubated with horseradish peroxidase-conjugated secondary antibody (Bio-Rad, Hercules, CA, USA). Images developed on the film were scanned using the Konica Minolta Bizhub C368 (Konica Minolta, Wayne, NJ, USA). Densitometry analysis was performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Gene expression of Amh and its receptors in the hippocampus

To determine gene expression of *Amh* and its receptors in the hippocampus, male ($n = 3$) and female ($n = 3$) mice were anesthetized with isoflurane and decapitated to obtain the brain. The hippocampal tissue was dissected out for RNA extraction using TRIzol (Thermo Fisher Scientific, Waltham, MA, USA), as previously described (19). RNA was reverse-transcribed into cDNA using a GoScript Reverse Transcription System (Promega Corporation, Madison, WI, USA) (20). PCR primers were designed using NCBI/Primer-BLAST (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) for *Amh*, *Amhr2* and *Alk2/3/6* (Table 1). PCR was conducted using GoTaq Green Master Mix (Promega Corporation) with the following parameters: initial denaturation 95 °C/1 min, followed by 36 cycles of denaturation 95 °C/30 s, annealing 58 °C/45 s, and extension 68 °C/45 s. The final extension

was at 72 °C for 3 min. PCR products were analyzed using gel electrophoresis and purified using a QIAquick PCR Purification Kit (QIAGEN, Valencia, CA, USA) for sequencing on a 3730xl DNA Analyzer (Thermo Fisher Scientific) by the Molecular Technologies Core at ONPRC, OHSU to verify their identity, as previously described (19). Sequencing results were analyzed using BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

To quantify mRNA levels of *Amh* and its specific receptor *Amhr2* in the hippocampus, real-time PCR was performed using cDNA obtained from the hippocampal tissue described above (n = 3 males and n = 3 females) and TaqMan Gene Expression Assays (*Amh* Assay ID: Mm00431795_g1 and *Amhr2* Assay ID: Mm00513847_m1; Thermo Fisher Scientific) on an Applied Biosystems 7900HT Fast Real-time PCR System (Thermo Fisher Scientific), as previously described (20). Mitochondrial ribosomal protein S10 (*Mrps10*) served as the internal control.

Protein expression of Amh and Amhr2 in the hippocampus

To assess protein expression levels of Amh and its specific receptor Amhr2 in the hippocampus, hippocampal tissue was collected from male (n = 3) and female (n = 3) mice for Western blot, as previously described (18). Briefly, total protein was extracted using a RIPA buffer containing 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, and 25 mM Tris-HCl (pH 7.6). Western blot and densitometry analysis were performed, as described above. The primary antibodies included anti-mouse Amh (1:1000; MAB1737; R&D Systems), Amhr2 (1:1000; MBS1496327; MyBioSource, San Diego, CA, USA) and α -Tubulin (loading control; 1:12000; T6074; Sigma-Aldrich) antibodies. The specificity of Amh and Amhr2 antibodies were characterized by immunohistochemistry using ovarian sections in previous studies (20) and preliminary experiments. Positive staining was only detected in granulosa cells of growing follicles expressing Amh and Amhr2. Antibodies preabsorbed with blocking peptides were used as negative controls.

Amh and Amhr2 protein expression was further localized in the hippocampus using immunofluorescence staining on mouse brain coronal frozen sections (n = 3 males or females; MF-201-08; Zyagen, San Diego, CA, USA), as previously described (6). Briefly, brain sections were blocked using 5% normal donkey serum (Sigma-Aldrich) in phosphate-buffered saline and then incubated with primary antibodies anti-mouse Amh (1:200; AF1446; R&D Systems) and Amhr2 (1:200; ab197148; Abcam, Cambridge, MA, USA) at 4 °C overnight. The specificity of Amh and Amhr2 antibodies were characterized by immunofluorescence using ovarian sections in preliminary experiments. Positive staining was only detected in granulosa cells of growing follicles expressing Amh and Amhr2. Antibodies preabsorbed with blocking peptides were used as negative controls. Sections were subsequently incubated with DyLight 594- and Fluorescein-conjugated secondary antibodies (Vector Laboratories, Burlingame, CA, USA) and DAPI (nuclear staining; Thermo Fisher Scientific). Images were captured using a Revolve microscope (Echo, San Diego, CA, USA).

Hippocampal slice preparation

Mice (numbers of animals detailed in Results for each experiment) were anesthetized with isoflurane and rapidly decapitated. The brain was removed, and 300 μm slices from the middle of the hippocampus were cut using a vibrating microtome (VT1200S; Leica Instrument, Leitz, Nussloch, Germany) while the brain was immersed in an ice-cold sucrose substituted artificial CSF (aCSF; control solution) with the following composition (in mM): 119 NaCl, 26 NaHCO_3 , 2.5 KCl, 1 NaH_2PO_4 , 1 MgCl_2 , 2 CaCl_2 , and 25 dextrose (oxygenated with a carbogen mixture of 95% O_2 and 5% CO_2). Slices were held in oxygenated aCSF at 35 $^\circ\text{C}$ for 30 min, and then at room temperature (22–24 $^\circ\text{C}$) for at least 1 h before the subsequent electrophysiological recordings.

Electrophysiological recordings

All extracellular field recordings were performed at 32 $^\circ\text{C}$. Hippocampal slices (number of slices detailed in Results for each experiment) were visualized using a fixed-stage upright microscope (Axioskop; Carl Zeiss, Thornwood, NY, USA) equipped with infrared differential interference contrast optics. The recording chamber was continuously perfused with aCSF (oxygenated with a carbogen mixture of 95% O_2 and 5% CO_2) flowing at a rate of 1–2 ml/min. Recording electrodes were pulled from borosilicate pipettes (Sutter Instruments), and had tip resistances of 2–3.5 M Ω when filled with aCSF for extracellular field recordings. Glass stimulating electrodes of approximate resistance of 1 M Ω were filled with aCSF, connected to a Digitimer constant current stimulus isolation unit (AutoMate Scientific, Berkeley, CA, USA), and placed in the middle of the CA1 stratum radiatum approximately 100 μm from the recording electrode to stimulate the CA3 axon collaterals. The CA3 region was severed to eliminate recurrent excitation within the CA3 subfield. The stimulating and recording electrodes were placed in the middle portion of the CA1 stratum radiatum (approximately equal distance from stratum pyramidal and stratum lacunosum moleculare). Stimulus duration was 0.1 ms allowing for clear separation of fiber volley (FV) from the preceding stimulus artifact. Long-term potentiation (LTP) of CA3–CA1 excitatory synapses was induced by stimulating CA3 axons with 3 sets of 100 stimuli delivered at 50 Hz. Recordings were obtained using a Multiclamp 700B amplifier (Molecular Devices, San Jose, CA, USA) in current clamp. Extracellular field potentials were filtered at 5 kHz, digitized at 20 kHz using an ITC-16 interface (InstruTech, Port Washington, NY, USA), and transferred to a computer using Patchmaster software (Heka, Holliston, MA, USA).

Whole-cell patch-clamp recordings were obtained from CA1 pyramidal cells using patch pipettes (open pipette resistance 2–4 M Ω) filled with (in mM) 133 K-gluconate, 4 KCl, 4 NaCl, 1 MgCl_2 , 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 4 MgATP, 0.3 Na_3GTP , and 10 K_2 -phosphocreatine (pH 7.3). All whole-cell patch-clamp recordings were made at room temperature. The stimulating electrode was positioned in the proximal CA1 stratum radiatum, ~150 μm away from the stratum pyramidale. EPSPs were evoked with synaptic stimulate of 1 ms in duration, delivered once every 20 s. Whole-cell recordings were obtained using a Multiclamp 700B amplifier (Molecular Devices, San Jose, CA, USA). Whole-cell recordings of excitatory postsynaptic potentials (EPSPs) were filtered at 3 kHz and digitized at 10 kHz. Series resistance was electronically compensated to greater than 70%. A bias current was applied to maintain the membrane potential in current clamp at –65

mV. The input resistance in current clamp was determined from a 20 pA hyperpolarizing pulse applied at the end of each sweep. All recordings were from cells with a stable input resistance.

Amh treatment for field recordings was achieved by soaking slices in aCSF containing 0.4 nM recombinant human Amh protein (rhAMH; 1737-MS, R&D Systems) prior to transferring to the recording chamber (6). Amh-treated slices were continuously perfused with 0.4 nM rhAMH in the recording chamber. For whole-cell recordings of EPSPs, rhAMH was added to the bath solution following a stable EPSP baseline recording. Additionally, in order to document the specificity of Amh effects, 10 μ M LDN193189 dihydrochloride (Tocris Bioscience, Minneapolis, MN, USA) was bath perfused for 10 min before rhAMH administration. LDN193189 dihydrochloride is a chemical compound that blocks Amh-activated downstream signaling by inhibiting type I receptors of bone morphogenetic proteins (21).

All experiments were performed in the presence of SR95531 (5 μ M) and CGP55845 (2.5 μ M) (Tocris Bioscience) to suppress inhibitory synaptic transmission.

Statistical analysis

ELISA, Western blot, and real-time PCR data were analyzed using an unpaired Student's *t*-test by SigmaPlot 11 software (SPSS, Chicago, IL, USA). Voltage traces were analyzed using custom macros written in Igor Pro software (WaveMetrics, Tigard, OR, USA). Data were compared statistically using a paired Student's *t*-test for whole-cell EPSP recordings or a linear mixed-effects model with a random effect for individual mice for field excitatory postsynaptic potential (fEPSP) recordings. Ensemble averages were compared statistically using a nonparametric Wilcoxon-Mann-Whitney two-sample rank test in Igor Pro. Custom macros written in Igor Pro are available on request (J.M.). Data are presented as mean $\pm$ SEM. $P < 0.05$ was accepted as statistically significant.

RESULTS

Amh is present in the blood and the CSF

Amh protein was detectable in all plasma samples from mice by ELISA. Plasma Amh concentrations were 81-fold higher ($p = 0.03$) in females compared with those of males (47.89 ± 17.01 versus 0.59 ± 0.23 ng/ml).

A ~75 kDa and a ~50 kDa protein bands were identified in CSF samples from mice by Western blot (Fig. 1A). The band sizes were consistent with the predicted band sizes of uncleaved (70 kDa) and cleaved (55 kDa) mouse Amh monomers, respectively. There were no differences in CSF levels of uncleaved ($p = 0.26$) or cleaved ($p = 0.13$) Amh monomers between male and female mice (Fig. 1B). The CSF levels of total Amh protein (uncleaved and cleaved) were also comparable between males and females (total Amh/albumin = 0.45 ± 0.02 versus 0.64 ± 0.15 ; $p = 0.14$).

Amh and its receptors are expressed in the hippocampus

PCR products with predicted band sizes (Table 1) were identified for *Amh*, *Amhr2* and *Alk2/3/6* in all hippocampal samples from mice (representatives in Fig. 1C). Amplicon identities were confirmed by sequencing, which yielded 99–100% nucleotide sequence match between PCR products and the respective mouse genes. Real-time PCR showed that *Amh* mRNA levels were 1.7-fold higher ($p = 0.002$) in the hippocampal samples of females than those of males (Fig. 1D). In contrast, mRNA levels of the ligand-specific receptor *Amhr2* were comparable ($p = 0.14$) between the hippocampal samples from male and female mice (Fig. 1D).

A ~75 kDa and a ~50 kDa protein bands (uncleaved and cleaved Amh), as well as a protein band (Amhr2) between 50 and 75 kDa, were identified in all hippocampal samples from mice (Fig. 1E). The band sizes were consistent with the predicted band sizes of mouse Amh and Amhr2 proteins, respectively. The total Amh protein (uncleaved and cleaved) levels were 2.4-fold higher ($p = 0.04$) in the hippocampal samples of females than those of males (Fig. 1F). The hippocampal levels of uncleaved (uncleaved Amh/ α -Tubulin = 0.41 ± 0.11 versus 0.06 ± 0.02 ; $p = 0.02$), but not the cleaved (cleaved Amh/ α -Tubulin = 0.73 ± 0.06 versus 0.42 ± 0.21 ; $p = 0.12$), Amh monomers were higher in females relative to males. In contrast, protein levels of the ligand-specific receptor Amhr2 were comparable ($p = 0.33$) between the hippocampal samples from male and female mice (Fig. 1F).

In the brain sections from all mice studied, positive immunostaining for Amh (red; representatives in Fig. 2A,E) and Amhr2 (green; representatives in Fig. 2B,F) proteins was detected in the CA1 (Fig. 2A,B) and CA3 (Fig. 2E,F) regions of the hippocampus. Both the cell body and dendrites of neurons in the CA1 region were Amh-positive (Fig. 2A,C inserts), while Amh-positive staining appeared only in the cell body of neurons in the CA3 region (Fig. 2E,G inserts). The expression patterns of Amhr2 were similar to those of Amh in the CA1 (Fig. 2B,C inserts) and CA3 (Fig. 2F,G inserts) regions. Positive staining of Amhr2 was co-localized with that of Amh in the cell body and dendrites of neurons (Fig. 2D,H inserts).

Amh increases excitatory synaptic strength

Field recordings were used to measure synaptic transmission and LTP on the same slices either in control aCSF or treated with 0.4 nM rhAMH for greater than 30 min. To quantify the strength of CA3-CA1 synaptic transmission, acute brain slices were prepared from mice and extracellular fEPSPs were evoked by synaptic stimuli of varying intensities. Increasing stimulus intensities resulted in increases in the FV and fEPSP for representative slices from the same mouse in control and rhAMH-containing solutions (Fig. 3A,B). The FV, measured as the peak inward deflection (FV in Fig. 3A,B), predominantly reflects the number of CA3 axon collaterals that fired an action potential. The FV versus stimulus intensity (Stim) relationship was well described by a linear regression, the slope of which reflects the intrinsic excitability and recruitment of CA3 axons (Fig. 3D). The slope of the FV-Stim relationship showed no significant differences between sexes or between the control and rhAMH-treated slices (21 control and 23 rhAMH-treated slices from 7 male mice; 40 control and 46 rhAMH-treated slices from 15 female mice; Fig. 3C). These results suggested that

intrinsic excitability and recruitment of CA3 axon collaterals were not affected by rhAMH exposure.

The initial slope of fEPSPs (vertical gray bar in Fig. 3A,B) primarily reflects activation of postsynaptic AMPA receptors (AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors). The fEPSP slope versus FV relationship was well described by a linear regression (Fig. 3E). The slope of fEPSP slope-FV relationship reflects the composite cellular transfer function between presynaptic action potential (AP)-evoked glutamate release and postsynaptic membrane response, which was well described by a linear regression without constraints (Fig. 3E). The slope of the regression line was used as an indicator of synaptic strength which was referred to as fEPSP input/output (I/O). Acute slices from female and male mice exposed to rhAMH displayed a robust increase in synaptic strength (fEPSP I/O) compared with their controls, respectively (Fig. 3F). For females, synaptic strength increased ($p < 0.001$) from $0.291 \pm 0.032 \text{ ms}^{-1}$ in controls (40 slices from 15 mice) to $0.606 \pm 0.061 \text{ ms}^{-1}$ in rhAMH-treated slices (46 slices from 15 mice). For males, synaptic strength increased ($p = 0.007$) from $0.429 \pm 0.053 \text{ ms}^{-1}$ in controls (21 slices from 7 mice) to $0.864 \pm 0.139 \text{ ms}^{-1}$ in rhAMH-treated slices (23 slices from 7 mice). On average, the fEPSP I/O slopes measured from rhAMH-treated slices were 2.1- and 2.0-fold larger than those of control slices in females and males, respectively.

The ensemble averages of the FV-Stim, fEPSP slope-Stim, and fEPSP slope-FV relationships for the male and female mice in Figure 3 are shown in Figure 4. The ensemble FV-Stim relationships were not different between the control and rhAMH-treated slices from either female or male mice (Fig. 4A,B). For confirmation of increased synaptic strength by rhAMH, the ensemble fEPSP slope versus stimulus intensity showed that rhAMH increased (p values shown in the figure legend) the fEPSP slope evoked at each stimulus intensity in both female and male mice (Fig. 4C,D). The slope of the fEPSP slope-Stim relationship in the rhAMH group was 2.4- and 2.0-fold larger than controls in female and male mice, respectively. The ensemble fEPSP I/O was generated by averaging the fEPSP slope for a FV bin interval of 0.25 mV (Fig. 4E,F). The ensemble fEPSP I/O demonstrated an increase (p values shown in the figure legend) in synaptic transmission by rhAMH treatment (Fig. 4E,F). Adjusted for sex, fEPSP I/O was $0.339 \pm 0.089 \text{ ms}^{-1}$ in controls and $0.610 \pm 0.086 \text{ ms}^{-1}$ in slices treated with rhAMH ($p < 0.001$ using a linear mixed-effects model of I/O with fixed effects for test condition, sex, an interaction of test condition and sex, and a random effect for individual mice). The current evidence is not sufficient to conclude a sex difference in excitatory synaptic strength altered by rhAMH treatment. These results demonstrated that the application of rhAMH resulted in a 1.8-fold increase in synaptic strength at the hippocampal CA3-CA1 synapse.

Acute Amh treatment does not affect glutamate release

The increase in fEPSP I/O slope in the presence of rhAMH may reflect Amh actions involving presynaptic and/or postsynaptic mechanisms. Paired-pulse ratio (PPR), defined as a ratio of the amplitude of the second fEPSP to that of the first after a closely delivered pair of stimuli, is a sensitive assay for presynaptic changes in probability of release (Pr) of glutamate (22). When paired-pulse stimulations separated by 50 ms were applied (Fig.

5A,C), slices exposed to rhAMH did not show altered PPR relative to controls in either female or male mice (13 females: 1.36 ± 0.22 from 45 slices versus 1.43 ± 0.23 from 35 slices, $p = 0.18$; 7 males: 1.41 ± 0.20 from 22 slices versus 1.45 ± 0.17 from 21 slices, $p = 0.48$) (Fig. 5B). The data showed that the boosting of synaptic transmission by rhAMH was not mediated by an increase in glutamate release at CA3-CA1 synapses.

Amh boosts long-term synaptic potentiation

The hippocampus is essential for memory formation, and LTP of the CA3-CA1 excitatory synapses is thought to be the cellular correlate that mediates hippocampus-dependent memory formation (23–25). To determine whether Amh alters LTP at these synapses, extracellular fEPSPs were measured at CA3-CA1 synapses following measurement of the I/O function (Fig. 3), with the initial stimulus intensity set to give 25–50% of the maximal field response. After achieving 5 minutes of stable baseline responses, a high frequency train of stimuli (3 sets at 0.08 Hz of 100 pulses at 50 Hz) was administered to induce LTP (Fig. 6A). The degree of LTP was measured 15–16 minutes after delivery of the high frequency train (Fig. 6A,B). Slices exposed to rhAMH showed increased levels of LTP compared with control slices in both female and male mice (12 females: 168.9 ± 7.0 % from 36 slices versus 140.8 ± 6.6 % from 31 slices, $p = 0.005$; 5 males: 208.6 ± 15.5 % from 17 slices versus 154.9 ± 7.5 % from 15 slices, $p = 0.005$) (Fig. 6C). The current evidence is not sufficient to conclude a sex difference in long-term synaptic potentiation altered by rhAMH treatment. Combining data from both sexes yielded an average LTP of 145.4 ± 5.1 % (46 slices) in controls and 181.6 ± 7.2 % (53 slices) in rhAMH-treated slices ($p = 0.005$). These results suggested enhanced information storage/coding in hippocampal slices following rhAMH exposure.

Amh acutely increases EPSPs at CA3-CA1 synapses

Because rhAMH did not acutely change intrinsic excitability of CA3 axon collaterals (Fig. 3C) or presynaptic glutamate release (Fig. 5B), the increase of the I/O slope in the presence of rhAMH (Fig. 3F) most likely reflected postsynaptic mechanisms. To verify Amh actions using cells as their own controls, we performed whole-cell current-clamp recordings of mixed AMPA and NMDA (N-methyl-D-aspartate) receptor-mediated EPSPs from CA1 pyramidal neurons in control solution followed by bath application of rhAMH. Neurons were maintained at -65 mV throughout the recordings with bias current injections. Bath application of rhAMH increased the amplitude of EPSPs in slices from both male and female mice within 20 min (Fig. 7A). Representative EPSPs recorded are shown in Figure 7B. The increases in EPSP by rhAMH exposure were 163.07 ± 16.13 % for females (18 slices from 9 mice, $p = 0.003$) and 177.09 ± 12.02 % for males (11 slices from 5 mice, $p = 0.0002$) (Fig. 7C). These results demonstrated that rhAMH acutely increased synaptic strength by increasing the postsynaptic membrane response to glutamate which was consistent with the boosting of fEPSPs by rhAMH treatment.

To determine whether the boosting of EPSPs was induced specifically by Amh, hippocampal slices were exposed to LDN193189 dihydrochloride throughout the recording to block Amh-activated downstream signaling. Bath application of rhAMH in the presence of LDN193189 did not change the amplitude of evoked EPSPs in 7 slices from 4 females ($p = 0.87$) (Fig.

7A,C). These results support the direct actions of Amh in regulating synaptic transmission and LTP that are mediated by intracellular signaling pathways activated via Amh binding to its receptors.

DISCUSSION

Using a mouse model, we studied the expression and direct actions of Amh in the hippocampus. Our central finding is that Amh, which is produced locally in the brain, markedly increases synaptic strength and long-term synaptic plasticity of the hippocampal neurons. The administration of exogenous Amh protein rapidly increased synaptic responses in hippocampal CA3-CA1 synapses in vitro in both male and female mice. This is the first evidence demonstrating the potential paracrine/autocrine actions of Amh in regulating synaptic transmission in the hippocampus.

For the first time, the presence of Amh protein is identified in the CSF from both male and female mammals. Because circulating Amh produced by the gonads does not cross the blood-brain barrier (14), the CSF Amh is predominantly generated locally in the central nervous system. Indeed, the plasma, but not the CSF, Amh levels were different between male and female mice in the current study. This suggests that the circulating and the central Amh levels may not correlate with each other among adult individuals. According to the current data, the hippocampus produces Amh which, if secreted, could enter the CSF via the interstitial fluid drainage from this major structure of the limbic system (15, 16). Therefore, hippocampal Amh has the potential to act as a paracrine factor via the CSF circulation in regulating functions of other brain structures, such as the hypothalamus which is known to express *Amhr2* (6). Although data are not yet available, it cannot be ruled out that Amh protein secreted from other areas of the brain could be collected into the CSF.

The current study is the first demonstration of mRNA and protein expression of Amh in the central nervous system. Amh protein is localized in/on neurons of the CA1 and CA3 regions, which suggests that Amh effects in the hippocampus could be paracrine/autocrine actions in nature. A recent clinical study in men with *Amh* single-nucleotide polymorphisms indicated significant associations between serum Amh levels and reasoning scores, reaction time, and episodic memory of patients, which were independent of age, education, and cardiovascular conditions (10). The evidence suggested a causal relationship between Amh and cognitive function, at least in males, though the circulating Amh levels measured in the study only indirectly reflected Amh levels in the central nervous system of individuals with or without genetic alterations. Furthermore, our data showed that the mRNA and protein levels of hippocampal Amh were higher in female than those of male mice. An exploratory study in children demonstrated that differences in the brain “maturity index” between boys and girls were associated with their serum Amh levels (13). It appeared that Amh could contribute to sex biases in the cognitive development, though the correlation between circulating Amh levels assessed in the study and the Amh levels in the central nervous system in those prepubertal individuals were not clear. Therefore, studies are warranted to further investigate Amh production and function in the hippocampus in males and females at different developmental stages.

Given that Amh receptors are expressed in the hippocampus, especially that the ligand-specific Amhr2 protein is present in the CA1 and CA3 regions, Amh can directly activate Amhr2-mediated downstream signaling to regulate hippocampal neuronal activities. Based on our data, Amhr2 was localized in the cell body and dendrites of CA1 neurons, but only in the cell body of CA3 neurons, which was consistent with the current electrophysiological study showing that Amh increased synaptic transmission and LTP at CA3-CA1 synapses in the hippocampus. To date, data are not available from the direct measurement of excitability of CA1 or CA3 pyramidal neurons induced by Amh. When cell-attached voltage recordings were conducted on mouse hypothalamic slices, exogenous Amh protein addition increased firing rates of the gonadotropin-releasing hormone expressing (GnRH) neurons, which altered the hormone secretion patterns of cells (6). Therefore, Amh effects on firing activity and excitability of CA1 and CA3 pyramidal neurons could be tested in future studies to access Amh actions on the cell bodies and terminals.

Using electrophysiological approaches, our functional studies indicate that Amh increases hippocampal synaptic transmission and plasticity. Synaptic transmission is the process involving the release of presynaptic neurotransmitter and the activation of postsynaptic neurotransmitter receptor, reflecting the first step of signal transfer between most of the central neurons. Altered synaptic transmission changes action potential generation in the postsynaptic neuron, as well as information storage within individual synapses depending on postsynaptic Ca^{2+} influx (26). Synaptic dysfunction and impaired synaptic plasticity at the hippocampal synapses are commonly observed in animal models of diseases or cognitive deficits, such as Alzheimer's disease (27) and accelerated aging (28). At CA3-CA1 synapses, a long-lasting increase in synaptic strength was shown to facilitate hippocampus-dependent learning and memory during acquisition, extinction, recall and reconditioning of an associative task (23, 25). Therefore, Amh may have a positive impact on synaptic activities and hippocampal function.

This is the first evidence showing that Amh enhances functions of the hippocampal neurons. Indeed, the treatment of rhAMH to acute hippocampal slices resulted in an increased fEPSP I/O slope, which primarily reflected an increased level of AMPA receptor activity evoked by a given number of CA3 axons firing an action potential. An increased I/O slope at the CA3-CA1 synapses could be due to an increase in glutamate release from presynaptic terminals or the postsynaptic sensitivity to glutamate, which could be mediated by an increased function and/or density of AMPARs (29, 30). We found that PPR, an indicator for Pr of glutamate at CA3-CA1 synapses, was unaffected by rhAMH treatment, suggesting a postsynaptic mechanism for Amh effects on synaptic I/O function. Consistently, the expression of LTP at CA3-CA1 synapses is thought to take place primarily in the postsynaptic neurons, and the boost of synaptic strength is largely accomplished by the rapid increase of synaptic AMPARs (31). Synaptic transmission increased by rhAmh treatment could not account for the increase in LTP as the increase in synaptic transmission occurred before induction of LTP. One model of LTP involves the activity-dependent recirculation of AMPARs in the postsynaptic density (PSD): exocytosis of AMPARs from perisynaptic sites to the plasma membrane, followed by a lateral translocation into the PSD (32, 33). In addition, recent evidence suggested that CA1 hippocampal synapses might also undergo the activation of GluA3-containing AMPARs during synaptic plasticity, switching the AMPARs from low- to

high-conductance state (34). The boosting effects of rhAMH on LTP suggest that Amh increases synaptic transmission via increasing AMPAR density-increased number of receptors and/or increasing AMPAR conductance. Alternatively, since postsynaptic potentials are mixed AMPAR and NMDAR, as well as postsynaptic ion channel activities, the increase in EPSP by rhAMH treatment could result from increased activities of AMPAR and NMDAR or decreased activities of postsynaptic ion channels such as the small-conductance Ca^{2+} -activated K^{+} channels (SK channels) and voltage-gated A-type $\text{Kv}4$ channels that shape dendritic EPSPs (35–38). Further studies are required to delineate the intracellular signaling pathways whereby Amh increases synaptic transmission and LTP.

The current study demonstrates novel effects of Amh as a central regulator of synaptic transmission in the hippocampus. Research is needed to further investigate the expression patterns of hippocampal Amh and Amhr2 at different stages of brain development, the mechanisms of Amh actions in regulating the hippocampal neuronal activities, and the physiological significance of central Amh paracrine/autocrine function at the systemic level. The information may provide insight into the potential role of Amh in learning and memory, and the possible cause of sex differences in cognitive development and function.

ACKNOWLEDGMENTS

We are grateful for Drs. Cadence True and Byung Park, Ms. Maralee Lawson, as well as members of the Division of Comparative Medicine, the Transgenic Mouse Model Core, the ONPRC Molecular Technologies Core and the Endocrine Technologies Core at OHSU for their valuable expertise and technical assistance. We thank Ms. Korin Riske at the Sunset High School for supporting science internship opportunities.

Research reported in this publication was supported by the National Institutes of Health (NIH) Office of the Director P51OD011092 and the NIH Eunice Kennedy Shriver National Institute of Child Health & Human Development (NICHD) R01HD082208. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

ABBREVIATIONS:

aCSF	artificial cerebrospinal fluid
Alk2/3/6	activin receptor-like kinase 2/3/6
Amh	anti-Müllerian hormone
Amhr2	anti-Müllerian hormone receptor 2
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
AP	action potential
CA	Cornu Ammonis
CSF	cerebrospinal fluid
EPSP	excitatory postsynaptic potential

fEPSP	field excitatory postsynaptic potential
FV	fiber volley
FV-Stim	fiber volley versus stimulus intensity
GluA3	glutamate ionotropic receptor AMPA type subunit 3
GnRH	gonadotropin-releasing hormone
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
I/O	input/output
Kv4 channel	Shal-type (Kv4.x) K ⁺ channel
LTP	long-term potentiation
Mrps10	mitochondrial ribosomal protein S10
NMDA	N-methyl-D-aspartate
OHSU	Oregon Health & Science University
ONPRC	Oregon National Primate Research Center
PPR	paired-pulse ratio
Pr	probability of release
PSD	postsynaptic density
rhAMH	recombinant human anti-Müllerian hormone protein
SK channel	Ca ²⁺ -activated K ⁺ channel
Stim	stimulus intensity
TGF-β	transforming growth factor beta family

REFERENCES

1. Münsterberg A and Lovell-Badge R (1991) Expression of the mouse anti-müllerian hormone gene suggests a role in both male and female sexual differentiation. *Development* 113, 613–624 [PubMed: 1782869]
2. Lee MM, Donahoe PK, Hasegawa T, Silverman B, Crist GB, Best S, Hasegawa Y, Noto RA, Schoenfeld D, and MacLaughlin DT (1996) Mullerian inhibiting substance in humans: normal levels from infancy to adulthood. *J Clin Endocrinol Metab* 81, 571–576 [PubMed: 8636269]
3. Josso N, Racine C, di Clemente N, Rey R, and Xavier F (1998) The role of anti-Mullerian hormone in gonadal development. *Mol Cell Endocrinol* 145, 3–7 [PubMed: 9922092]
4. Dewailly D, Andersen CY, Balen A, Broekmans F, Dilaver N, Fanchin R, Griesinger G, Kelsey TW, La Marca A, Lambalk C, Mason H, Nelson SM, Visser JA, Wallace WH, and Anderson RA (2014) The physiology and clinical utility of anti-Mullerian hormone in women. *Hum Reprod Update* 20, 370–385 [PubMed: 24430863]
5. Lebeurrier N, Launay S, Macrez R, Maubert E, Legros H, Leclerc A, Jamin SP, Picard JY, Marret S, Laudonbach V, Berger P, Sonderegger P, Ali C, di Clemente N, and Vivien D (2008) Anti-

- Mullerian-hormone-dependent regulation of the brain serine-protease inhibitor neuroserpin. *J Cell Sci* 121, 3357–3365 [PubMed: 18796535]
6. Cimino I, Casoni F, Liu X, Messina A, Parkash J, Jamin SP, Catteau-Jonard S, Collier F, Baroncini M, Dewailly D, Pigny P, Prescott M, Campbell R, Herbison AE, Prevot V, and Giacobini P (2016) Novel role for anti-Müllerian hormone in the regulation of GnRH neuron excitability and hormone secretion. *Nat Commun* 7, 10055
 7. di Clemente N, Josso N, Gouédard L, and Belville C (2003) Components of the anti-Müllerian hormone signaling pathway in gonads. *Mol Cell Endocrinol* 211, 9–14 [PubMed: 14656470]
 8. Wang PY, Protheroe A, Clarkson AN, Imhoff F, Koishi K, and McLennan IS (2009) Müllerian inhibiting substance contributes to sex-linked biases in the brain and behavior. *Proc Natl Acad Sci U S A* 106, 7203–7208 [PubMed: 19359476]
 9. Sweatt JD (2004) Hippocampal function in cognition. *Psychopharmacology (Berl)* 174, 99–110 [PubMed: 15205881]
 10. Nelson SM, Iliodromiti S, Pell J, and Lyall D (2018) Association of anti-müllerian hormone with cognitive function in Uk BioBank: a mendelian randomization study. *Fertil Steril* 110 Supplement, e106
 11. Pankhurst MW and McLennan IS (2012) Inhibin B and anti-Müllerian hormone/Müllerian-inhibiting substance may contribute to the male bias in autism. *Transl Psychiatry* 2, e148
 12. Harden CL, Pennell PB, French JA, Davis A, Lau C, Llewellyn N, Kaufman B, Bagiella E, and Kirshenbaum A (2016) Anti-mullerian hormone is higher in seizure-free women with epilepsy compared to those with ongoing seizures. *Epilepsy Res* 127, 66–71 [PubMed: 27565413]
 13. Morgan K, Ruffman T, Bilkey DK, and McLennan IS (2017) Circulating anti-Müllerian hormone (AMH) associates with the maturity of boys' drawings: does AMH slow cognitive development in males? *Endocrine* 57, 528–534 [PubMed: 28593614]
 14. Tata B, Mimouni NEH, Barbotin AL, Malone SA, Loyens A, Pigny P, Dewailly D, Catteau-Jonard S, Sundström-Poromaa I, Piltonen TT, Dal Bello F, Medana C, Prevot V, Clasadonte J, and Giacobini P (2018) Elevated prenatal anti-Müllerian hormone reprograms the fetus and induces polycystic ovary syndrome in adulthood. *Nat Med* 24, 834–846 [PubMed: 29760445]
 15. Szentistványi I, Patlak CS, Ellis RA, and Cserr HF (1984) Drainage of interstitial fluid from different regions of rat brain. *Am J Physiol* 246(6 Pt 2), F835–F844 [PubMed: 6742132]
 16. Bedussi B, Naessens DMP, de Vos J, Olde Engberink R, Wilhelmus MMM, Richard E, Ten Hove M, vanBavel E, and Bakker ENTP (2017) Enhanced interstitial fluid drainage in the hippocampus of spontaneously hypertensive rats. *Sci Rep* 7, 744 [PubMed: 28389645]
 17. Lim NK, Moestrup V, Zhang X, Wang WA, Møller A, and Huang FD (2018) An improved method for collection of cerebrospinal fluid from anesthetized mice. *J Vis Exp* 133
 18. Cabrera-Socorro A, Pueyo Morlans M, Suarez Sola ML, Gonzalez Delgado FJ, Castañeyra-Perdomo A, Marin MC, and Meyer G (2006) Multiple isoforms of the tumor protein p73 are expressed in the adult human telencephalon and choroid plexus and present in the cerebrospinal fluid. *Eur J Neurosci* 23, 2109–2118. [PubMed: 16630058]
 19. Xu J, Stouffer RL, Searles RP, and Hennebold JD (2005) Discovery of LH-regulated genes in the primate corpus luteum. *Mol Hum Reprod* 11, 151–159 [PubMed: 15723857]
 20. Xu J, Xu F, Letaw JH, Park BS, Searles RP, and Ferguson BM (2016) Anti-Müllerian hormone is produced heterogeneously in primate preantral follicles and is a potential biomarker for follicle growth and oocyte maturation in vitro. *J Assist Reprod Genet* 33, 1665–1675 [PubMed: 27638727]
 21. Hayata T, Chiga M, Ezura Y, Asashima M, Katabuchi H, Nishinakamura R, and Noda M (2018) Dullard deficiency causes hemorrhage in the adult ovarian follicles. *Genes Cells* 23, 345–356 [PubMed: 29521016]
 22. Katz B and Miledi R (1968) The role of calcium in neuromuscular facilitation. *J Physiol* 195, 481–492 [PubMed: 4296699]
 23. Gruart A, Muñoz MD, and Delgado-García JM (2006) Involvement of the CA3-CA1 synapse in the acquisition of associative learning in behaving mice. *J Neurosci* 26, 1077–1087 [PubMed: 16436593]

24. Pastalkova E, Serrano P, Pinkhasova D, Wallace E, Fenton AA, and Sacktor TC (2006) Storage of spatial information by the maintenance mechanism of LTP. *Science* 313, 1141–1144 [PubMed: 16931766]
25. Whitlock JR, Heynen AJ, Shuler MG, Bear MF (2006) Learning induces long-term potentiation in the hippocampus. *Science* 313, 1093–1097 [PubMed: 16931756]
26. Cavazzini M, Bliss T, and Emptage N (2005) Ca^{2+} and synaptic plasticity. *Cell Calcium* 38, 355–367 [PubMed: 16154476]
27. Chapman PF, White GL, Jones MW, Cooper-Blacketer D, Marshall VJ, Irizarry M, Younkin L, Good MA, Bliss TV, Hyman BT, Younkin SG, and Hsiao KK (1999) Impaired synaptic plasticity and learning in aged amyloid precursor protein transgenic mice. *Nat Neurosci* 3, 271–276
28. Lopez-Ramos JC, Jurado-Parras MT, Sanfeliu C, Acuna-Castroviejo D, and Delgado-Garcia JM (2012) Learning capabilities and CA1-prefrontal synaptic plasticity in a mice model of accelerated senescence. *Neurobiol Aging* 33, e13–e26
29. Malinow R and Tsien RW (1990) Presynaptic enhancement shown by whole-cell recordings of long-term potentiation in hippocampal slices. *Nature* 346, 177–180 [PubMed: 2164158]
30. Branco T and Staras K (2009) The probability of neurotransmitter release: variability and feedback control at single synapses. *Nat Rev Neurosci* 10, 373–383 [PubMed: 19377502]
31. Kerchner GA and Nicoll RA (2008) Silent synapse and the emergence of a postsynaptic mechanism for LTP. *Nat Rev Neurosci* 9, 813–825 [PubMed: 18854855]
32. Yang Y, Wang XB, Frerking M, and Zhou Q (2008) Delivery of AMPA receptors to perisynaptic sites precedes the full expression of long-term potentiation. *Proc Natl Acad Sci U S A* 105, 11388–11393 [PubMed: 18682558]
33. Yudowski GA, Puthenveedu MA, Leonoudakis D, Panicker S, Thorn KS, Beattie EC, and von Zastrow M (2007) Real-time imaging of discrete exocytic events mediating surface delivery of AMPA receptors. *J Neurosci* 27, 11112–11121 [PubMed: 17928453]
34. Renner MC, Albers EH, Gutierrez-Castellanos N, Reinders NR, van Huijstee AN, Xiong H, Lodder TR, and Kessels HW (2017) Synaptic plasticity through activation of GluA3-containing AMPA-receptors. *eLife* 6, e25462
35. Ngo-Anh TJ, Bloodgood BL, Lin M, Sabatini BL, Maylie J, and Adelman JP (2005) SK channels and NMDA receptors form a Ca^{2+} -mediated feedback loop in dendritic spines. *Nat Neurosci* 8, 642–649 [PubMed: 15852011]
36. Lin MT, Lujan R, Watanabe M, Adelman JP, and Maylie J (2008) SK2 channel plasticity contributes to LTP at Schaffer collateral-CA1 synapses. *Nat Neurosci* 11, 170–177 [PubMed: 18204442]
37. Wang K, Lin MT, Adelman JP, and Maylie J (2014) Distinct Ca^{2+} sources in dendritic spines of hippocampal CA1 neurons couple to SK and Kv4 channels. *Neuron* 81, 379–387 [PubMed: 24462100]
38. Kim J, Jung S-C, Clemens AM, Petralia RS, and Hoffman DA (2007) Regulation of dendritic excitability by activity-dependent trafficking of the A-type K^{+} channel subunit Kv4.2 in hippocampal neurons. *Neuron* 54, 933–947 [PubMed: 17582333]

Figure 1.

The presence of anti-Müllerian hormone (Amh) in the mouse cerebrospinal fluid (CSF), and the expression of Amh and its receptors in the mouse hippocampus. *A*) The uncleaved (70 kDa) and cleaved (55 kDa) Amh monomers were detected in the CSF of male ($n = 3$) and female ($n = 3$) mice, as determined by Western blot. *B*) The CSF levels of uncleaved and cleaved Amh monomers were comparable between males and females by densitometry analysis. Albumin served as the loading control. *C*) The mRNAs of *Amh*, the ligand-specific type II receptor (*Amhr2*), and activin receptor-like kinase 2/3/6 (*Alk2/3/6* encoding type I

receptors of bone morphogenetic proteins) were expressed in the mouse hippocampus (representative animal). *D*) The hippocampal mRNA levels of *Amh*, but not *Amhr2*, were higher in the females (n = 3) compared with those of males (n = 3), as determined by real-time PCR. Mitochondrial ribosomal protein S10 (*MRPS10*) served as the internal control. *E*) Proteins of Amh and the ligand-specific type II receptor (Amhr2) were expressed in the hippocampus of male (n = 3) and female (n = 3) mice, as determined by Western blot. *F*) The hippocampal protein levels of total Amh (uncleaved and cleaved monomers), but not Amhr2, were higher in females compared with those of males by densitometry analysis. The α -Tubulin served as the loading control. *, significant difference between males and females, $p < 0.05$. Data are presented as the mean $\pm$ SEM.

Figure 2.

Protein localization of anti-Müllerian hormone (Amh) and the ligand-specific type II receptor (Amhr2) in the mouse hippocampus. Coronal sections were fluorescently labeled for Amh (red; *A,E*), Amhr2 (green; *B,F*) and DAPI (nuclear staining; blue; *C,G*). A high magnification image of the boxed area is shown in the top-left insert for each panel. *A,E*) Amh-positive staining was detected in the Cornu Ammonis (CA) 1 and CA3 regions. *B,F*) Amhr2-positive staining was detected in the CA1 and CA3 regions. *D,H*) Amh- and Amhr2-

positive staining was co-localized in cell bodies and dendrites (arrows). Scale bar = 40 μm for panels *A-H* and 10 μm for inserts.

Figure 3.

Acute effect of anti-Müllerian hormone (Amh) on synaptic transmission in the mouse hippocampus. *A,B*) Representative extracellular field excitatory postsynaptic potential (fEPSP) evoked by increasing stimulation intensities from hippocampal slices of the same mouse in the control (*A*) and 0.4 nM recombinant human AMH protein (rhAMH) (*B*) solution. Each trace represents an average of 5 consecutively recorded voltage traces. Gray bars highlight regions where fEPSP initial slopes were measured. Fiber volley (FV) indicates peak FV. The stimulus artifact preceding the FV was blanked out. *C*) Scatter plot of slope of FV versus stimulus strength (Stim) relationship from slices in control solution (control) or treated with rhAMH (rhAMH) from female (40 control and 46 rhAMH-treated slices from 15 mice) and male (21 control and 23 rhAMH-treated slices from 7 mice) mice. *D*) FV versus Stim (FV-Stim) relationship for the control and rhAMH groups. The FV-Stim relationships were fit with linear functions without constraints yielding a slope of 0.0145

mV/ μ A (control) and 0.0138 mV/ μ A (rhAMH). *E*) fEPSP slope versus FV relationship. The fEPSP slope-FV relationships were fit with linear functions without constraints. The slope derived from the fits reflects the input-output relation of synaptic transmission (fEPSP I/O) of 0.337 s⁻¹ (control) and 1.007 s⁻¹ (rhAMH). *F*) Scatter plot of fEPSP I/O from slices in control solution (control) or treated with rhAMH (rhAMH). ***p* < 0.01 and ****p* < 0.001.

Figure 4.

Anti-Müllerian hormone (Amh) increases ensemble average of (fEPSP) slope and fEPSP synaptic input/output (I/O) in the hippocampus from mice described in Figure 2A. ,B) Ensemble average of fiber volley (FV)-stimulus strength (Stim) relationship for females (A) and males (B). Data are presented as mean $\pm$ SEM for hippocampal slices in the control and 0.4 nM recombinant human AMH protein (rhAMH) solution. The ensemble FV-Stim relationships were well described by a linear regression without constraints yielding a slope of 0.0109 (control) and 0.0114 mV/ μ A (rhAMH) for females, and 0.0119 (control) and

0.0112 mV/ μ A (rhAMH) for males. *C,D*) Ensemble average of fEPSP slope versus Stim relationship for females (*C*) and males (*D*). Data were binned for each Stim intensity and presented as mean $\pm$ SEM. Significance was determined for each Stim bin using a nonparametric Wilcoxon-Mann-Whitney two-sample rank test. The fEPSP slope-Stim relationships were well described by a linear regression without constraints between 20–80 μ A yielding a slope of 0.0029 (control) and 0.0071 mV/ μ A (rhAMH) for females, and 0.0046 (control) and 0.0090 mV/ μ A (rhAMH) for males. *E,F*) fEPSP slope versus FV relationships for females (*E*) and males (*F*). Data were binned at 0.25 mV FV intervals and presented as mean $\pm$ SEM for control and rhAMH groups. Significance was determined for each FV bin using a nonparametric Wilcoxon-Mann-Whitney two-sample rank test. The ensemble fEPSP-FV relationships were well described by a linear regression without constraints yielding a slope of 0.290 (control) and 0.576 ms⁻¹ (rhAMH) for females, and 0.357 (control) and 0.773 ms⁻¹ (rhAMH) for males. **p* < 0.05, ***p* < 0.01 and ****p* < 0.001.

Figure 5.

Anti-Müllerian hormone (Amh) does not affect paired-pulse ratio (PPR) in the mouse hippocampus. *A*) Average of 10 consecutive extracellular field excitatory postsynaptic potential (fEPSP) pairs (50 ms inter-pulse interval) from a representative hippocampal slice of a female mouse. *B*) Scatter plot of PPR in control hippocampal slices (control) and slices exposed to 0.4 nM recombinant human AMH protein (rhAMH) for female (35 control and 45 rhAMH-treated slices from 13 mice) and male (21 control and 22 rhAMH-treated slices from 7 mice) mice. *C*) Overlay of first (black) and second (red) fEPSP trace evoked by 50 ms paired-pulse stimulus interval in the representative hippocampal slice from panel *A*. Gray bar highlights the region where fEPSP initial slopes were measured and compared.

Figure 6.

Anti-Müllerian hormone (Amh) increases long-term synaptic plasticity (LTP) formation in the mouse hippocampus. *A, B*) Time course (mean $\pm$ SEM) of the extracellular field excitatory postsynaptic potential (fEPSP) response (slope) normalized to baseline before ($-2-0'$) and after ($0-16'$) LTP induction for hippocampal slices from female (*A*) and male (*B*) mice in the control (control) and 0.4 nM recombinant human AMH protein (rhAMH) solution. Insert above demonstrates a 50 Hz stimulation train and typical response elicited by LTP stimulation protocol (3×100 pulses at 50 Hz). The solid traces on the right are representative averages of 10 fEPSPs from individual experiments before (baseline, black) and after (magenta) LTP induction. Gray bars highlight regions where fEPSP initial slopes were measured. *C*) Scatter plot of LTP depicting the increased levels of LTP observed in rhAMH-treated slices (rhAMH) versus controls (control) for female (31 control and 36 rhAMH-treated slices from 12 mice) and male (15 control and 17-rhAMH treated slices from 5 mice) mice. $**p < 0.01$.

Figure 7.

Anti-Müllerian hormone (Amh) acutely increases excitatory postsynaptic potential (EPSP) in the mouse hippocampus, and the Amh effect on boosting EPSP is blocked by an Amh receptor inhibitor. *A*) Summary time course plots of normalized EPSP amplitude of hippocampal slices in the control solution for females and males, and in 10 μ M LDN193189 dihydrochloride solution for females (LDN, Amh receptor inhibitor). Bath application of 0.4 nM recombinant human AMH protein (rhAMH) was applied at 0–20'. The rhAMH addition did not increase EPSP in the presence of LDN. Data are binned into 1 min intervals. The voltage traces used for averaging were taken at time points highlighted with gray and red shadings. *B*) Representative voltage traces (average of 15 EPSPs) acquired in the control solution (baseline, black) and in the presence of rhAMH (magenta) for individual neurons. *C*) Summary scatter plot of normalized EPSP amplitude in the presence (7 slices from 4 female mice) or absence (18 slices from 9 female and 11 slices from 5 male mice) of LDN. ** $p < 0.01$ and *** $p < 0.001$

TABLE 1.

PCR primers

Gene	Forward	Reverse	Length
<i>Amh</i>	GACCCCTTCCTAGAGACCCT	CTCCGGGATGAGCACTGAAC	573 bp
<i>Amhr2</i>	GGAGGCTTCAGGGTGAGATG	TGTACCCCAAGTCCTGCAAGT	551 bp
<i>Alk2</i>	AAACCACCCTAACCTCGCTC	AGGGCCACAGTGCAAGTAAG	299 bp
<i>Alk3</i>	GACACTGCCCAGATGATGCT	TAGCGGCCTTTACCAACCTG	541 bp
<i>Alk6</i>	GTTACGGCCTTCATTCCCA	CCTACCAGTGACACCAACCC	778 bp

Amh, anti-Müllerian hormone; *Amhr2*, Amh receptor II; *Alk2/3/6*, activin receptor-like kinase 2/3/6.