HHS Public Access

Author manuscript

Mol Psychiatry. Author manuscript; available in PMC 2015 September 08.

Published in final edited form as:

Mol Psychiatry. 2015 April ; 20(4): 490–499. doi:10.1038/mp.2014.47.

Role of the Vasopressin 1b Receptor in Rodent Aggressive Behavior and Synaptic Plasticity in Hippocampal Area CA2

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Abstract

The vasopressin 1b receptor (Avpr1b) is critical for social memory and social aggression in rodents, yet little is known about its specific roles in these behaviors. Some clues to Avpr1b function can be gained from its profile of expression in the brain, which is largely limited to the pyramidal neurons of the CA2 region of the hippocampus, and from experiments showing that inactivation of the gene or antagonism of the receptor leads to a reduction in social aggression. Here we show that partial replacement of the Avpr1b through lentiviral delivery into the dorsal CA2 region restored the probability of socially motivated attack behavior in total Avpr1b knockout mice, without altering anxiety-like behaviors. To further explore the role of the Avpr1b in this hippocampal region, we examined the effects of Avpr1b agonists on pyramidal neurons in mouse and rat hippocampal slices. We found that selective Avpr1b agonists induced significant potentiation of excitatory synaptic responses in CA2, but not in CA1 or in slices from Avpr1b knockout mice. In a way that is mechanistically very similar to synaptic potentiation induced by oxytocin, Avpr1b agonist-induced potentiation of CA2 synapses relies on NMDA receptor activation, calcium and calcium/calmodulin-dependent protein kinase II activity, but not on cAMP-dependent protein kinase activity or presynaptic mechanisms. Our data indicate that the hippocampal CA2 is important for attacking in response to a male intruder and that the Avpr1b, likely through its role in regulating CA2 synaptic plasticity, is a necessary mediator.

Introduction

Aggressive behavior, found across the animal kingdom, is usually highly adaptive for survival. Examples include maternal protection of the young, defense of territory, and capture of prey[1, 2]. Human aggressive behavior, on the other hand, particularly that involving physical violence, is typically viewed as pathological and can accompany several

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Conflict of interest: The authors declare no conflict of interest.

forms of psychiatric illness such as schizophrenia. An understanding of these complex behaviors at the cellular level, however, is still lacking. With the goal of ameliorating some symptoms of mental illness, including aggressive behavior, many recent human studies have explored the effects of the two neuropeptides vasopressin (Avp) and oxytocin (Oxt)[3]. Many of the effects of these neuropeptides are likely mediated by the oxytocin (Oxtr) and vasopressin 1a (Avpr1a) receptors[4-6] which are widely distributed within the central nervous system. Vasopressin 1b receptor (Avpr1b) expression, in contrast, is highly restricted to the pyramidal cells in the CA2 region of the hippocampus[7]. Like the Oxtr and Avpr1a, this receptor is likely to play an important role in human behavior, as it is required for proper regulation of social aggression and social recognition in several mammalian species[8, 9].

The CA2 region was described in 1934[10], yet little is known of its function as it had, until recently, been overlooked as part of the hippocampal circuit (reviews[11, 12]). Pathological studies show that compared to neurons in the flanking CA1 and CA3 fields, neurons in CA2 are relatively resistant to damage arising during the course of various illnesses, including epilepsy[13-18]. Conversely, CA2 non-pyramidal neurons in schizophrenic and bipolar patients seem to be preferentially lost[19] and pyramidal neurons in CA2 of schizophrenics are smaller[20]. Gene expression studies show that the CA2 region is molecularly distinct from the rest of the hippocampus[21, 22]. Additionally, its pyramidal neurons have distinct physiological characteristics that include an apparent *lack* of capacity for typical long-term synaptic potentiation (LTP) when using conventional methods of Schaffer collateral stimulation[23]. This property of LTP resistance seems to be due to higher calcium buffering and extrusion and the expression of RGS14 in CA2 pyramidal neurons[24, 25].

To gain an understanding of its role in the brain, we inactivated the Avpr1b gene in the mouse and found that social recognition is reduced in both male and female mice in these knockouts (KO)[9, 26]. Specific inactivation of CA2 pyramidal cell activity also reduces social recognition[27]. In addition, male territorial aggression and maternal aggression are disrupted in this knockout line[9, 28]. Spatial memory, as tested in the Morris water maze, is normal[9]. Predatory aggression and defensive behaviors are unaffected, indicating that the pattern of motor skills important for aggressive behaviors remains intact[28]. Interestingly, although phenotypic changes in aggression often co-occur with changes in anxiety-like behavior[29], reduced aggression in the Avpr1b KOs is observed without any detectible changes in anxiety-like behavior[7]. Support for the role of Avpr1b in aggression comes from the administration of an Avpr1b antagonist to mice and hamsters that reduces aggressive behavior in these species[30, 31].

To test our hypothesis that Avp acts through the Avpr1b specifically expressed in dorsal CA2 to permit normal social aggression, we used lentiviral injections to partially restore Avpr1b expression there. This resulted in significant aggressive behavior in Avpr1b KO mice, without altering anxiety-like behavior. We then sought to determine the functional effects of Avp on CA2 synapses *in vitro* by testing our hypothesis that Avpr1b agonists modulate synaptic strength or plasticity in mice and rats. We found that application of the Avpr1b agonists induced significant potentiation of synaptic responses, an effect mimicked by Oxt and not found in Avpr1b knockout mice.

Materials and methods

Animals

The development and genotyping of the Avpr1b and Oxtr KO lines were described in detail previously[9, 32]. Briefly, Avpr1b mice were genotyped using these primers: 5, ACCTGTAGATATTTGACAGCCCGG; 9, GAAACGGCTACTCTCTCCGATTCCAAAAGAAAG; and neo1, ACCCCTTCCCAGCCTCTGAGCCCAGAAAGCGAAGG. PCR [95°C × 4′;(95°C × 1′, 60°C × 1′, 72°C × 1′) × 40; 72°C × 5′; 10°C] yields 762 and 461 bp bands for WTs and Avpr1b KOs, respectively (see Supplemental Figure 1 for schematic of the Avpr1b KO). Oxtr mice were genotyped using these primers: Seq. 1, ACCCCAGGAAGATGTACCCGTAGTAAAGC; Seq4, TTAGGTCCCAGGAAAGAGTCAGCCGCTCTGCCTGCAGAGAGG; and DTAo10.3, TGGGAGTCCAGAGATAGTGGAA. PCR [95°C × 4′, (95°C × 45″, 60°C × 45″, 72°C × 45″) × 40 72°C × 5, 10°C] yields bands at 221 and 150 bp for WTs and Oxtr KOs, respectively. The mice have been backcrossed into C57Bl/6J mice (Jackson Laboratories, Bar Harbor, ME) for more than ten generations and used here for in vivo behavior and *in vitro* electrophysiology experiments. Oxtr KO mouse pups were fostered with CD-1 dams (Charles River Laboratories, bred at NIEHS) until used for *in vitro* experiments.

Aggression experiments used a total of 48 male mice taken from 19 liters run in five separate squads: 16 wildtype (WT) mice, 19 KO mice injected with a lentivirus containing a cytomegalovirus promoter-driven mouse Avpr1b receptor coding sequence (KO+Replace) and 13 KO mice injected with a lentivirus containing a cytomegalovirus promoter-driven green fluorescent protein construct (KO+GFP). KO mice were randomly assigned to either the GFP or Avpr1b groups. All mice were between 81 and 216 days old at testing. A total of 42 BalbC mice obtained from the National Cancer Institute (Frederick, MD) at 8 weeks of age were used as intruders. Anxiety-like behavioral experiments used a total of 39 male mice run in 5 separate squads: 14 WT, 8 KO+GFP, and 8 KO+Replace. Tissues for brain slice experiments were obtained from mice or rats of either sex at 14-20 days of age, or 6 weeks of age in some experiments.

All animals were grouped housed in same sex cages (not segregated by genotype) from weaning until singly-housed for aggression, or housed with dams (*in vitro* studies) and maintained in a 12-hour light/dark cycle (lights on at 0400) with food and water available ad libitum. Behavioral tests were conducted one hour after the onset of the dark phase (1700 hours), and at least 2 weeks after surgery. All animal procedures were approved by the National Institute of Mental Health and National Institute of Environmental Health Sciences Animal Care and Use Committees and were in accordance with the National Institutes of Health guidelines on the care and use of animals.

Virus

The coding region of the Mus musculus Avpr1b mRNA (Gene Bank accession number: NM_011924) was cloned from mouse hippocampal tissue by RT-PCR. This region was inserted into the plasmid pRRLsin.CMV.GFPpre[33] replacing the GFP sequence. A flag tag

sequence (amino acids DYKDDDK) was also inserted after the ATG codon (Supplemental Figure 2a). A western blot was conducted to confirm proper mouse Avpr1b expression after infection of 293T cells (Supplemental Figure 2b). Vectors to express the lentiviral gag-pol and the VSV-G envelope were co-transfected into 293T cells and lentiviruses expressing GFP or Avpr1b were produced as previously described[33]. Titers of 1.7-2.2 × 10^8 particles per ml were obtained.

Surgical Procedures

Mice were anesthetized with 250-500 mg/kg intraperitoneal injections of 2,2,2-tribromoethanol (Avertin; Sigma-Aldrich, St. Louis, MO) and were kept warm with a heating pad during anesthesia and recovery. Each mouse was placed in a small animal stereotaxic instrument (Benchmark Angle Two, Leica Microsystems, Richmond, VA), and an ophthalmic ointment was applied to prevent drying of the eyes. The skull hair was plucked, the surgical area was disinfected, and a small incision was made to expose the skull. The skull was then opened over the appropriate stereotaxic coordinates using a small-burr (0.45mm) drill and a 30 gauge blunt-end needle was inserted for injection. Following needle removal, the skin was closed with wound clips. The mouse received 0.35 ml of warm saline intraperitoneally and was observed until waking. Then he was returned to the home cage and monitored carefully for the next 48 hours. Surgery was conducted in accordance with NIH Guidelines for rodent survival following surgery.

Virus Injections

Each mouse received pressure injections targeting six sites (three on each side) of the dorsal hippocampus of either lenti-Avpr1b or lenti-GFP (KO+Replace or KO+GFP, respectively) using a Hamilton syringe with a 30G blunt-ended needle mounted on a stereotaxic device. The viral solutions were stored at -80°C before being thawed on ice and backfilled into the syringes. The six sites of injection (per mouse) were: injection sites 1 and 2 medial-lateral (ML) relative to midline: ±1.25mm, anterior-posterior (AP) relative to bregma: -1.22, and dorsal-ventral (DV) relative to surface of the brain: -1.73mm; sites 3 and 4 ML: ±2.00, AP: -1.70, and DV: -1.83mm; and sites 5 and 6 ML: ±2.60, AP: -2.18, and DV: -2.15mm. The injection volume for each site was 400nl delivered at 50 nl/minute.

In Situ Hybridization Histochemistry (ISHH)

After behavioral testing, mice were killed and the brains were collected and frozen on dry ice. Viral expression of the Avpr1b in 16μm thick sections was determined by ISHH as described previously[7]. Images were obtained using a Cyclone phosphorimaging system (PerkinElmer, Waltham, MA) after 2-4 weeks of exposure. When we examined the ISHH results after the 6 bilateral lentiviral injections (3 each side, each one approximately 0.5 mm further posterior in A-P axis than the preceding one) for Avpr1b, we saw a spread that appears rather planar extending about 0.3 to 0.6 mm varying from site to site and from animal to animal (Figure 1). The CA2 region that expresses Avpr1b is in the dorsal (rostral third) hippocampus and extends about 1.5 mm in the A-P axis[7]. So, with a width of about 0.25mm for CA2, except most anteriorly where it is wider before CA1 appears, we estimate that even with the smaller injections as judged by the ISHH, we covered 60% (0.9mm of 1.5mm) of the CA2. With the larger injections, we approached 100%.

Aggression Experiments

Aggression was conducted as previously reported by our lab[9, 28]. All experimental mice were singly housed for two weeks, with a cage change one week prior to the encounter with the intruder. Stimulus mice were group housed and used for only a single encounter per day. No stimulus mouse was used for more than two aggressive episodes. The encounter took place in the experimental mouse's (resident's) home cage (32×17.5×14cm) with the wire rack for food and water removed. Encounters were recorded under red lighting with a Panasonic HDC-TM700 camera (Amazon.com) and later analyzed using the Observer XT software (version 10, Noldus Information Technology, Leesburg, VA) on a Dell personal computer.

Mice were brought into an anteroom and weighed just prior to lights out (1700 hours), and were then transported to the behavioral room and left to sit for one hour. A weight-matched intruder mouse was introduced into the resident's cage. If, after five minutes, no aggression occurred, the intruder was removed and a latency of 300 seconds was recorded. Aggression was allowed to continue for 2 minutes after its onset, which was defined by an attack. All interactions were recorded and scored at a later time. The latency to attack, attack duration, number of bites and number of tail rattles were measured and analyzed.

Anxiety-like Behavioral Tests

Anxiety-like behaviors were tested in an elevated ◯ maze (EOM; San Diego Instruments, San Diego, CA) and an open field. All animals were handled and tail-marked for identification the day prior to testing. Testing took place in a dark room with the open areas of the maze illuminated to 120 lux at the maze surface with soft white light. Mice were moved into an anteroom of the behavioral suite at 1700 hours and tests began at 1800 hours. Mice were placed on the open arm of the EOM (inside wall of diameter 20 inches) facing a closed arm and were allowed to explore the maze for 5 minutes.

The open field activity was measured as previously described[32, 34, 35]. On the test day, mice were brought to the testing room at least 30 minutes after lights off and allowed to sit in their cages for at least 30 minutes. Mice housed in the same cage were tested simultaneously. The Plexiglas testing apparatus had 4 identical arenas, which were 43 × 43 cm with 2 opaque walls and 2 clear outer walls. This apparatus was placed in the middle of the test room. The center was defined as the inner 32 × 32cm square. Each arena was evenly illuminated at 120-150 lux using white incandescent lights. The chambers were cleaned with 70% ethanol 5 minutes before each test session. Each mouse was placed in an open arena at the outermost corner facing the center and allowed to explore for 10 minutes.

Mice were recorded using a ceiling-mounted camera connected to a Dell computer running Ethovision software (Noldus Information Technology). Trials began as soon as the experimenter left the room. The distance traveled, and duration in and frequency of entry into the open arms or center of the arena are reported. These mice were also tested for aggression after the tests for anxiety-like behavior were run.

Electrophysiology

Slice preparation and recording techniques were described previously[23, 25, 36]. Hippocampal slices were prepared from mice (C57Bl/6J WT, Avpr1b KO, or Oxtr KO) or Sprague-Dawley rats (Charles River Laboratories, Raleigh, NC) of either sex. Under deep anesthesia with pentobarbital, animals were decapitated and the brains rapidly removed. Coronal brain slices (350μm thick) containing the hippocampus were cut using a vibrating blade microtome in ice-cold sucrose-substituted artificial cerebrospinal fluid (ACSF) containing (in mM): 240 sucrose, 2.0 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, and 10 glucose that was bubbled continuously with 95% O_2/5% CO_2 to obtain a pH of 7.4. Freshly cut slices were placed in a holding chamber with ACSF containing (in mM): 124 NaCl, 2.5 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, and 15 glucose and allowed to recover for at least one hour. For electrophysiological recordings, slices were then transferred to a recording chamber where they were bathed continuously with ACSF at room temperature (~24°C) at a rate of 2 ml/min. Whole-cell recordings from CA1, CA2, or CA3 neurons were made with patch pipettes (3-5 MΩ) filled with solution containing (in mM): 120 K-gluconate, 10 KCl, 3 $MgCl_2$, 0.5 EGTA, 40 HEPES, 2 ATP, 0.3 GTP, with pH adjusted to 7.2 with NaOH. Single test pulses were delivered via a single cluster-style electrode placed in the stratum radiatum to stimulate the Schaffer collaterals once every 15 seconds and evoke excitatory post synaptic currents (EPSCs) in CA2 or CA1. In experiments that recorded from CA3 neurons, EPSCs were evoked by stimulation of the commissural and associational CA3/CA3 fibers. Stimulation of the mossy fiber synapses was avoided in recordings from both CA2 and CA3 neurons. Following at least 5 minutes of stable recordings of baseline EPSCs, one of two Avpr1b agonists (10 nM D3PVP; 50 nM d[Leu^4,Lys^8]-Avp) or Oxt agonists (1 μM Oxt or 100 nM [Thr^4,Gly^7]-Oxt) were added to the bathing media for either 15 minutes or until the end of the 30-minute recording session. To assess agonist-induced changes in presynaptic function, effects on paired pulse facilitation for [Thr^4,Gly^7]-Oxt and d[Leu^4,Lys^8]-Avp, two pulses were delivered 20, 40, 80, 100, or 200 ms apart, and the ratio of the amplitudes of the second responses and the first responses calculated before and after drug application. Action potential threshold was determined in current clamp mode by depolarizing neurons and measuring the membrane potential at which the first action potentials occurred (rheobase current). Input resistance was calculated by measuring the amplitude of the steady-state current evoked during a -10 mV voltage step delivered 100 ms prior to test stimulation. No significant changes were observed in paired-pulse facilitation, action potential threshold, or input resistance between pre-drug baseline levels and those measured at 25 to 30 minutes post-drug. The effects of pharmacological treatments were assessed on the amplitude of averaged EPSCs obtained during 5-minute epochs recorded before and 10 minutes after drug application (last 5 minutes of the experiment). Because the CA3 is too close to CA2 to cut off without risking damage to the recording area, all slices were kept intact. As a result, epileptiform activity was observed in some of the experiments testing for the effect of bicuculline (experiments for Figure 4f). These cases were excluded from the dataset if the activity interfered with accurate measurements of EPSCs (5 of 21 total).

Chemicals

All chemicals were purchased from Tocris Bioscience (R&D Systems, Minneapolis MN) except where indicated. D3PVP [deamino-Cys^1, D-3-(pyridyl)-Ala^2, Arg^8-Avp] and [dLeu^4,Lys^8]-Avp were used as selective Avp1b receptor agonists. Oxt and [Thr^4,Gly^7]-Oxt (American Peptide Co., Sunnyvale, CA) were used as agonists of the Oxtr. The following drugs were used to interfere with known regulators of synaptic plasticity: BAPTA [1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid], a selective calcium chelator; KN-62 [4-[(2S)-2-[(5-isoquinolinylsulfonyl)methylamino]-3-oxo-3-(4-phenyl-1 piperazinyl)propyl] phenyl isoquinolinesulfonic acid ester], and KN-93 [*N*-[2-[[[3-(4-chlorophenyl)-2-propenyl]methylamino]methyl]phenyl]-*N*-(2-hydroxyethyl)-4-methoxybenzenesulphonamide], cell-permeable inhibitors of calcium/calmodulin-dependent protein kinase II alpha (CaMKII); PKI [protein kinase A (PKA) inhibitor fragment (6-22) amide], an inhibitor of the catalytic subunit of cAMP-dependent protein kinases; D-(-)-2-Amino-5-phosphonopentanoic acid (AP5), a competitive N-methyl-D-aspartic acid (NMDA) receptor antagonist; and bicuculline, a competitive antagonist of $GABA_A$ receptors. KN-92 [2-[N-(4-methoxybenzenesulfonyl)]amino-N-(4-chlorocinnamyl)-N-methylbenzylamine, monohydrochloride] is an inactive analog of KN-93 (Santa Cruz Biotechnology, Dallas, TX).

Statistical Analyses

For aggression studies, we compared the proportions of animals in each group that exhibited aggressive behaviors, Fisher's Exact tests were used. Normally distributed data from the EOM and open field tests were analyzed with one-way ANOVAS. For electrophysiological studies, all data are expressed as the mean ± SEM and are normalized to baseline recordings for plotting. Changes in response properties were assessed using paired samples t-tests.

Results

We first sought to ascertain whether the restoration of Avpr1b to only the CA2 could affect the aggression deficit that accompanies the total and constitutive elimination of the Avpr1b in KO mice. We studied three groups of mice: WTs and KOs targeted with expression in the CA2 of either GFP (KO+GFP) or Avpr1b (KO+Replace) using lentiviruses. Only the WT and KO+Replace mice had Avpr1b expression in the CA2 (Figures 1a,b). Similar to our previous findings with Avpr1b KO mice[9], only one of 12 KO mice that received GFP attacked the intruder compared to six of 16 Avpr1b replaced mice and nine of 16 WTs. The percentage of mice attacking was analyzed using Fisher's Exact Probability test. Using two 2×2 matrices, we found a significant difference between the WT and KO+GFP ($p<0.05$), but no difference between the WT and KO+Replace groups ($p>0.05$). This indicates a successful restoration of the attack behavior in the mice with Avpr1b replacement covering the CA2 (Figure 1c).

The groups differed in the latency to attack, which was analyzed by a one-way ANOVA [202±21, 284±24, and 256±21s for WT, KO+GFP and KO+Replace, respectively; $F(2,41)= 3.54$, $p<0.05$]. Post hoc testing with Tukey's HSD test demonstrated that the WT attacked significantly faster than the KO+GFP ($p<0.05$) but but the KO+Replace group was not

significantly different from either WT or KO+GFP groups ($p>0.05$). Thus the receptor replacement group had an intermediate attack latency. We observed no significant differences in attack durations (21.2±4.0 vs. 8.9±4.9; $F(1,13) = 3.80$, $p=0.073$) or the number of attacks (7.1±1.2 vs. 3.5±1.5; $F(1, 13) = 3.67$, $p=0.074$), but did see a difference in tail rattles (7.1±1.2 vs. 2.8±1.4; $F(1, 13) = 5.23$, $p<0.05$) for WT compared to KO+Replace, respectively. (Because only a single KO+GFP mouse attacked, this group could not be included in these analyses.)

To ensure that changes in aggression were not tied to changes in anxiety-like behavior or that the Avpr1b expression did not affect other behaviors, we tested a second group of mice on the open field and elevated ◯ maze (see Supplementary Table 1). No differences in anxiety-like behavior were observed between groups. Specifically, in the open field test, one-way ANOVAs revealed no difference in locomotor activity ($F(2, 29)=0.39$, $p=0.68$), frequency of entries into the central area ($F(2, 29)=0.11$, $p=0.90$), or center durations ($F(2,29)=0.77$, $p=0.47$). Similarly, in the EOM, no differences in locomotor activity ($F(2, 2931)=0.48$, $p=0.62$), frequency of entries ($F(2,2931)=0.42$, $p=0.66$) or duration in the open arm ($F(2, 29)=0..75$, $p=0.48$) were observed. The same pattern of aggression as seen in the first cohort above was observed in these mice tested after the anxiety-like behaviors were measured.

After showing that partial Avpr1b replacement restores aggression, we examined whether Avp influenced synaptic or cellular properties of CA2 pyramidal cells, where the receptor is highly expressed (Figure 2a). Using whole cell recordings of rat CA2 pyramidal cells *in vitro*, we found that application of 50 nM d[Leu^4,Lys^8]-Avp (n=9) or 50 nM D3PVP (n=7) induced a significant potentiation of EPSCs. Synaptic responses were facilitated to 194.2±21.8% ($t8=7.90$, $p<0.001$) and 153.0±15.0% ($t6=4.14$, $P<0.01$) of the baseline amplitude, respectively (Figure 2b; Supplementary Figure 3a). In addition, d[Leu^4,Lys^8]-Avp induced a significant and lasting potentiation of EPSCs in area CA2 in slices taken from adult animals (to 173.0±29.4% of baseline; n=7; $t12=2.73$, $P<0.05$; Supplementary Figure 3b). Neither compound was effective at inducing potentiation in CA1 neurons at these concentrations (d[Leu^4,Lys^8]-Avp, 101.5±13.8% of baseline, n=6; D3PVP, 98.8±11.2% of baseline, n=5). Consistent with the observation that (postsynaptic) CA2 pyramidal neurons express the Avpr1b, but not the (presynaptic) CA3 neurons[7], we found that d[Leu^4,Lys^8]-Avp potentiated only the AMPA receptor-dependent component of the EPSCs, but not the NMDA receptor-dependent component (Figures 3a, b); the AMPA/NMDA ratio was larger after peptide treatment, but was not significantly (Figure 3c). Furthermore, we found no significant effect of d[Leu^4,Lys^8]-Avp on paired-pulse facilitation, and there was a consistent increase in the coefficient of variation ($1/CV^2$) which accompanied the agonist-induced potentiation of EPSCs (Figures 3d-f). The lack of an effect on paired-pulse facilitation and the reduction in response-to-response variability (increase in $1/CV^2$) suggests that d[Leu^4,Lys^8]-Avp is acting postsynaptically to facilitate synaptic transmission in area CA2. We also found no significant change in membrane properties, such as input resistance (Supplementary Figures 4a, c) nor the number of spikes fired in response to suprathreshold current injection (Supplementary Figures 4a, b), nor any change in action potential threshold or rheobase current (109.09±11.24 and 114.55±11.47 pA for

baseline and during agonist, respectively; n=11). Together these results indicate that vasopressin modulates synaptic strength in CA2 neurons and that CA2 may be unique among hippocampal regions in that regard.

Oxytocin has received intensive study in humans for deficits in social behavior, yet effects of the peptide on synaptic responses in the hippocampus are subtle[37]. Given its similarity to the Avp peptide, and that the Oxtr binding is highly enriched in the CA2 and CA3 regions of C57Bl/6J mice (Figure 2d)[38] we sought to compare the effects of Avp with Oxt in CA2. Consistent with the Oxtr binding, we found that 100 nM [Thr^4,Gly^7]-Oxt mimicked the effects of Avp1b agonists in rat CA2 pyramidal neurons in that it induced a robust potentiation of excitatory synaptic currents (to 196.3±40.9% of baseline, n=11, t10=2.42, $p<0.05$; Figure 2e, red circles). In addition, and again consistent with the distribution of the receptor binding, this Oxtr agonist also induced synaptic potentiation in CA3 neurons (to 207.7±29.9%, n=7, t6=4.11, $p<0.01$; Figure 2e, green triangles), but had no significant effect on excitatory synaptic responses in CA1 at this concentration (Figure 2e, blue squares, n=8).

To determine definitively whether these agonists were acting on the intended receptors, we performed similar experiments using hippocampal slices prepared from Avpr1b and Oxtr KO mice (Figures 2c,f). As we observed using rat tissue, the agonists induced potentiation in CA2 neurons from wildtype mice (Avpr1b agonist, to 160.0±20.1% of baseline, n=6, t5=3.14, $p<0.05$, Figure 2c; Oxtr agonist, to 168.6±23.1% of baseline, n=7, t6=3.20, $p<0.05$, Figure 2f), but not in slices from the respective KOs (Avpr1b KO, n=7; Oxtr KO, n=6). Likewise, the agonists were still effective in the mice lacking the other receptor (Figures 2c, f: Avpr1b agonist in Oxtr KO mice, EPSCs enhanced to 225.5±49.7% of baseline, n=4; Oxt agonist in Avpr1b KO mice, EPSCs enhanced to 168.9±24.6% of baseline, n=11), further indicating that these peptides were acting selectively at their respective receptors.

Both Oxtr and Avpr1b signal through G-protein coupled receptors (GPCRs) of the G_q type, which are coupled to phospholipase C-dependent pathways that increase intracellular calcium levels through inositol triphosphate[39]. It was not clear, however, how these receptor agonists could cause any synaptic plasticity in CA2 given that typical LTP is not observed there due in large part to robust calcium handling processes[24]. Therefore, to better understand the mechanisms underlying Avpr1b- and Oxtr-induced synaptic potentiation, we investigated whether known pharmacological blockers of synaptic plasticity could inhibit these phenomena.

We found that the potentiating effect induced by Avpr1b and Oxtr agonists strongly resembled long-term potentiation (LTP) mechanistically (Figures 4a, Supplementary Figure 5a): effects of both drugs were blocked by an inhibitor of NMDA receptors (50 μM AP5; n=9 and n=6, respectively) and appeared to require synaptic stimulation (both n=7), with likely release of glutamate, during the drug application (Figure 4b, Supplementary Figure 5b). Furthermore, the potentiating effects required postsynaptic calcium (15 mM BAPTA in pipette; both n=5) and activity of the calcium and calmodulin dependent kinase CaMKII (10 μM KN-62 in pipette; n=7 and n=10, respectively; Figure 4c, Supplementary Figure 5c; and 1 μM KN-93 but not 10 μM KN-92, n=6 and n=7, respectively; Figure 4d). Unlike the potentiating effects of adenosine receptor antagonists like caffeine[36], however, the Avpr1b

and Oxtr agonist effects were unaffected by an inhibitor of the cAMP-dependent protein kinase, protein kinase A (20 μM PKI in pipette plus Avpr1b agonist, to 203.3±27.6% of baseline, n=8, $t7 = 2.72$, $p<0.05$, Figure 4e; plus Oxtr agonist, to 232.2±42.7% of baseline, n=9, $t8=4.76$, $p<0.01$, Supplementary Figure 5d). Moreover, the effects of the Avpr1b agonist did not appear to be working through or require $GABA_A$ receptors in that the potentiation was still observed when $GABA_A$ receptors were blocked with 10 μM bicuculline (Figure 4f) and that the Avpr1b agonist had no significant effect on isolated inhibitory postsynaptic currents (IPSCs; Supplementary Figure 3c). We therefore conclude that Avpr1b and Oxtr activation allows for an LTP-like enhancement of excitatory synaptic currents to be induced simply with baseline frequencies of stimulation (one test pulse delivered every 15 seconds).

Discussion

The hippocampus has long been known to regulate aggression. For example, removal of the cat hippocampus leads to a less aggressive, “placid” state[40]. Electrical stimulation of the cat dorsal or ventral hippocampus fails to elicit predatory attacks on rats. However, the latencies to hypothalamically-induced attacks are increased or decreased after paired dorsal or ventral hippocampal stimulations, respectively[41, 42]. Conversely, dorsal or ventral hippocampal lesions in the cat causes reduced or increased aggression from hypothalamic stimulations, respectively[43]. Large hippocampal lesions in rats similarly reduce shock-induced intra-species male aggression[44, 45]. In addition to reducing male aggression, these lesions also impair the development of social hierarchies in mice[46]. Interestingly, in humans, a history of aggression in borderline personality disorder is associated with reductions in hippocampal volume[47, 48]. Aggression often accompanies dementia and so, not surprisingly, it is also correlated with increased neurofibrillary tangles in the hippocampus[49]. These previous studies considered the hippocampus a homogenous region, and so specific roles for the CA1, CA2, and CA3 in aggression are not clear. Therefore, as the CA3 and CA1 are primary inputs and outputs of CA2, respectively, disruption of any of these areas would be expected to disrupt the functional output of CA2.

Extensive studies from our group and others show that vasopressin impacts aggression (see review[50]). Most previous studies suggest that Avp acts in limbic areas such as the anterior hypothalamus, presumably through the Avpr1a, to influence aggression[51]. The Avpr1a is found only at low levels, if at all, throughout the rodent dorsal hippocampus, however[38, 52-56]. Consistent with the deficits in the Avpr1b KO mice, systemic administration of an Avpr1b antagonist reduces aggression in hamsters[30] and mice[57], further supporting a role for the Avpr1b in aggression. Pharmacological blockade of Avpr1b has also been shown to act as an anxiolytic when administered peripherally to rats and mice[58, 59], but this seems to be dependent on pituitary function[60]. Although differences in anxiety-like behavior can greatly impact the probability of aggressive behavior[29, 61], Avpr1b KO mice do not differ from wild-type mice when assessed for anxiety-like behavior[7].

Vasopressin is present in the dorsal hippocampus[62], but its source to the CA2 area has been unclear. Albreck and coworkers[63] suggested that Avp, released from terminals of axons originating in the medial amygdala, diffuses to the dorsal hippocampus. Recently, however,

Avp projections from the paraventricular nucleus (PVN) of the hypothalamus of the mouse[64] and rat[65] into the CA2 were discovered. The PVN receives and responds to convergent physiological and psychological inputs[66] and is thus well positioned to exert an integrated influence on CA2 neurons.

Avpr1b is enriched in the CA2, so we hypothesized that the Avpr1b there is responsible for the profound deficit in social aggression in the total KO[9]. Here we tested whether restoration of receptor expression in CA2 alone was sufficient to restore a cognitive function that was impaired in the total KO. If so, that would rule out a critical role for the pituitary corticotrophs (where this receptor was first discovered)[67] or the few cells elsewhere in the central nervous system that express Avpr1b. Our results show that expression of Avpr1b in the CA2 alone is sufficient to enable the Avpr1b KO mouse to exhibit social aggression. Viral replacement did not completely establish normal levels of aggression, but we note that the injections, placed in six different sites within the CA2, may not have covered the entire dorsal CA2. Also, although we cannot rule out the possibility that the Avpr1b in the CA2 has an important developmental (perhaps organizational) function in addition to its role in adults, our finding that Avpr1b introduced later in adulthood can restore aggressive behavior argues against a critical developmental role for Avpr1b in the circuitry formation. Importantly, we confirmed that the increased aggressive behavior seen in KO+Replace mice was not caused by a change in an anxiety-like phenotype. Furthermore, social memory is reduced in the Avpr1b KO[9] and following chronic inhibition of CA2 synaptic transmission[27]. These findings support the idea that social recognition, as mediated by the Avpr1b in he CA2, is a cognitive ability critical for the expression of appropriate social aggression. Interestingly, in humans, polymorphisms of the Avpr1b are associated with childhood aggression[68], autistic traits[69], and a protective effect against recurrent major depression[70]. Also, CA2 non-pyramidal neurons in schizophrenics seem to be preferentially lost[19] and pyramidal neurons in CA2 of schizophrenics are smaller[20]. It is possible that the paranoia that often accompanies this illness is produced by inappropriate evaluation of the social situation resulting in an expectation of impending aggression. Thus, the Avpr1b within the CA2 may be involved in several psychiatric diseases with social components. Further characterization of these polymorphisms with respect to the protein function is clearly needed.

Findings have varied in the few studies examining the effects of vasopressin on synaptic transmission in the hippocampus, with a tendency toward excitation[71-77]. Most studies, however, did not focus on the CA2 where the Avpr1b is most highly expressed. Given that the both Avpr1b and Oxtr are linked to phospholipase C and calcium increases[39], and that LTP is calcium-dependent[78], we propose that Avp and Oxt dramatically decrease the threshold for LTP in CA2 principle neurons. Our data showing that even baseline frequencies of stimulation can induce potentiation during agonist application, and that the potentiation is sensitive to NMDA receptor blockers, a calcium chelator, and CaMKII inhibitors (Figure 4a), support this idea. As such, these results suggest that the peptides could be more effective treatments in humans if paired with specific behavioral interventions. Further, these findings point to a mechanism for potentiation that is decidedly different than that induced in CA2 by caffeine and other A1 adenosine receptor antagonists, which a) relies on cAMP and PKA activity and b) appears not to require synaptic

stimulation for its appearance[36]. Importantly, these data suggest that the CA2 Schaffer collateral synapses are highly regulated by neuromodulatory substances at concentrations that do little at other hippocampal synapses.

Previously, we showed that Avpr1b KO mice have intact attack motor skills when defending themselves or attacking prey[28] as well as intact main olfaction and olfactory pathway activation[9], despite reduced interest in social odors (presumably accessory olfactory information)[79]. Here, we find that the CA2 Avpr1b receptor enables appropriate social aggression and significant potentiation of CA2 synaptic responses. We suggest a model in which social chemosensory information obtained by investigating another mouse travels via components of the accessory olfactory system to the entorhinal cortex and then onto the CA2 area of the hippocampus (this model does not preclude the input of additional information arriving from other areas such as the supramammillary nucleus). Simultaneously, this social interaction with the other mouse activates vasopressinergic neurons within the hypothalamic paraventricular nucleus to release Avp in the CA2. Avp activation of the Avpr1b then enhances synaptic potentiation leading to association (or, subsequently, recall) of social circumstances with specific odors. These social circumstances would include spatial context and behavior of the other mouse. We speculate that this coincidental input arrives at CA2 neurons that, in a fashion analogous to place cells in the CA1 hippocampus, are social cells that will form associations and respond to similar social situations. As opposed to place cells in which olfactory cues may aid in their stabilization[80, 81], olfactory cues would be essential for the establishment of the social “space” that the CA2 neurons encode. This framework should be testable, for example, by measuring the activities of CA2 pyramidal neurons in freely behaving mice exposed to various and repeated social situations. Future studies of the CA2, including investigations of the role of the Avpr1b there, should provide exciting new insights into how such a small region may have profound influences on social behavior.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank Emily Shepard and June Song for their technical assistance as well as the NIMH Animal Program and NIEHS Comparative Medicine Branch. This research was supported by the Intramural Research Program of the National Institutes of Health, National Institute of Mental Health (Z01-MH-002498-24) and National Institute of Environmental Health Sciences (Z01 100221).

Support: This research was supported by the NIMH (Z01-MH-002498-24) and the NIEHS (Z01-ES-100222) Intramural Research Programs.

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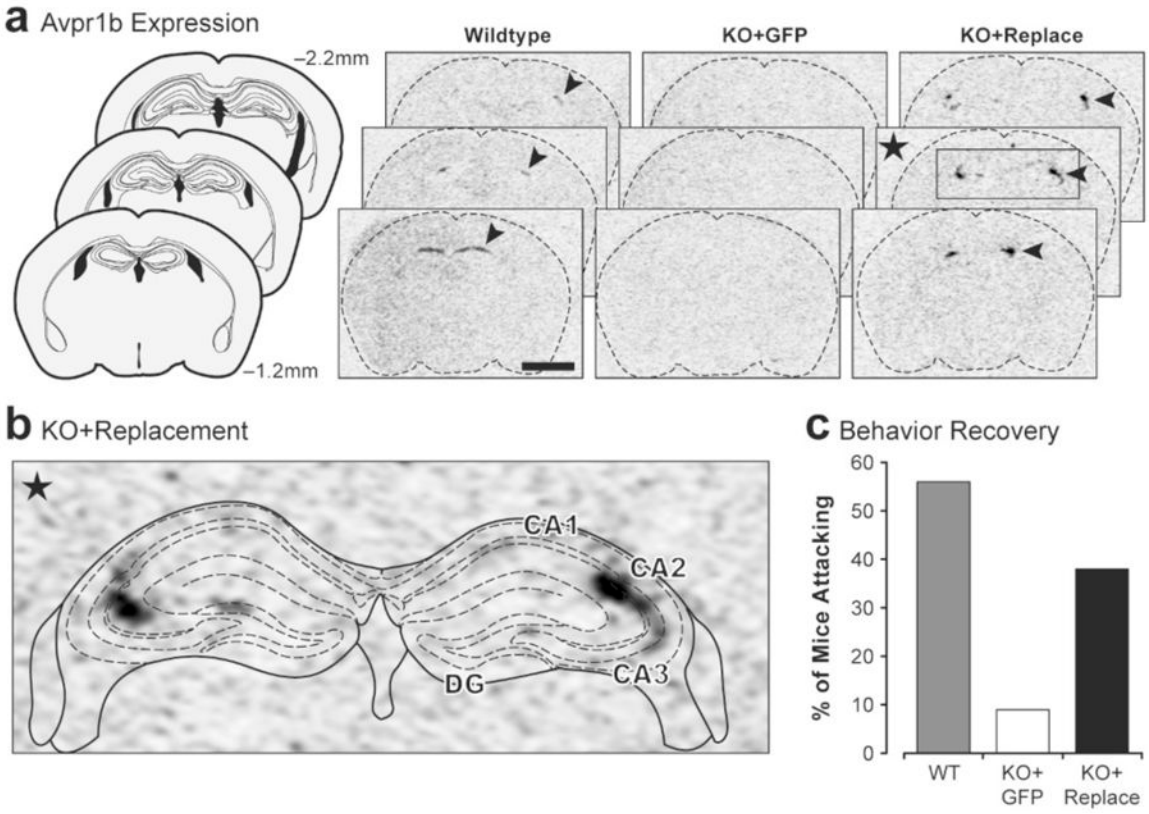

Figure 1.

Aggressive behavior is rescued in Avpr1b KO mice following bilateral injections of a lentivirus expressing the mouse Avpr1b into hippocampal area CA2. **(a)** The schematic diagrams on the left depict the mouse hippocampus at the three levels targeted for bilateral lentiviral replacement (at coordinates 1.2 to 2.2 mm posterior to the bregma). Image plates are adapted from a brain atlas[82]. Images on the right highlight the expression of Avpr1b in the hippocampus detected by *in situ* hybridization histochemistry in a representative WT mouse (Wildtype), a Avpr1b KO mouse injected with a GFP control lentivirus (KO+GFP), and a KO mouse injected with the Avpr1b lentivirus (KO+Replace). Arrowheads indicate examples of high Avpr1b expression in the hippocampus (calibration bar = 2 mm). **(b)** An expanded view of the lentiviral expression from the boxed region in the image marked with a star in **(a)** is presented with the hippocampal cells layers superimposed. Note that the high Avpr1b expression from the lentivirus localized bilaterally at the injection sites in area CA2. **(c)** Aggressive behavior was restored in Avpr1b KO mice following bilateral injections of the Avpr1b lentivirus into area CA2 (KO+Replace), but not in mice injected with a virus to express GFP (KO+GFP). Behavior data are from 16 WT mice, 13 KO mice injected with a lentivirus containing a cytomegalovirus promoter-driven green fluorescent protein (CMV-GFP) construct (KO+GFP), and 19 KO mice injected with a lentivirus containing a construct for the Avpr1b (KO+Replace).

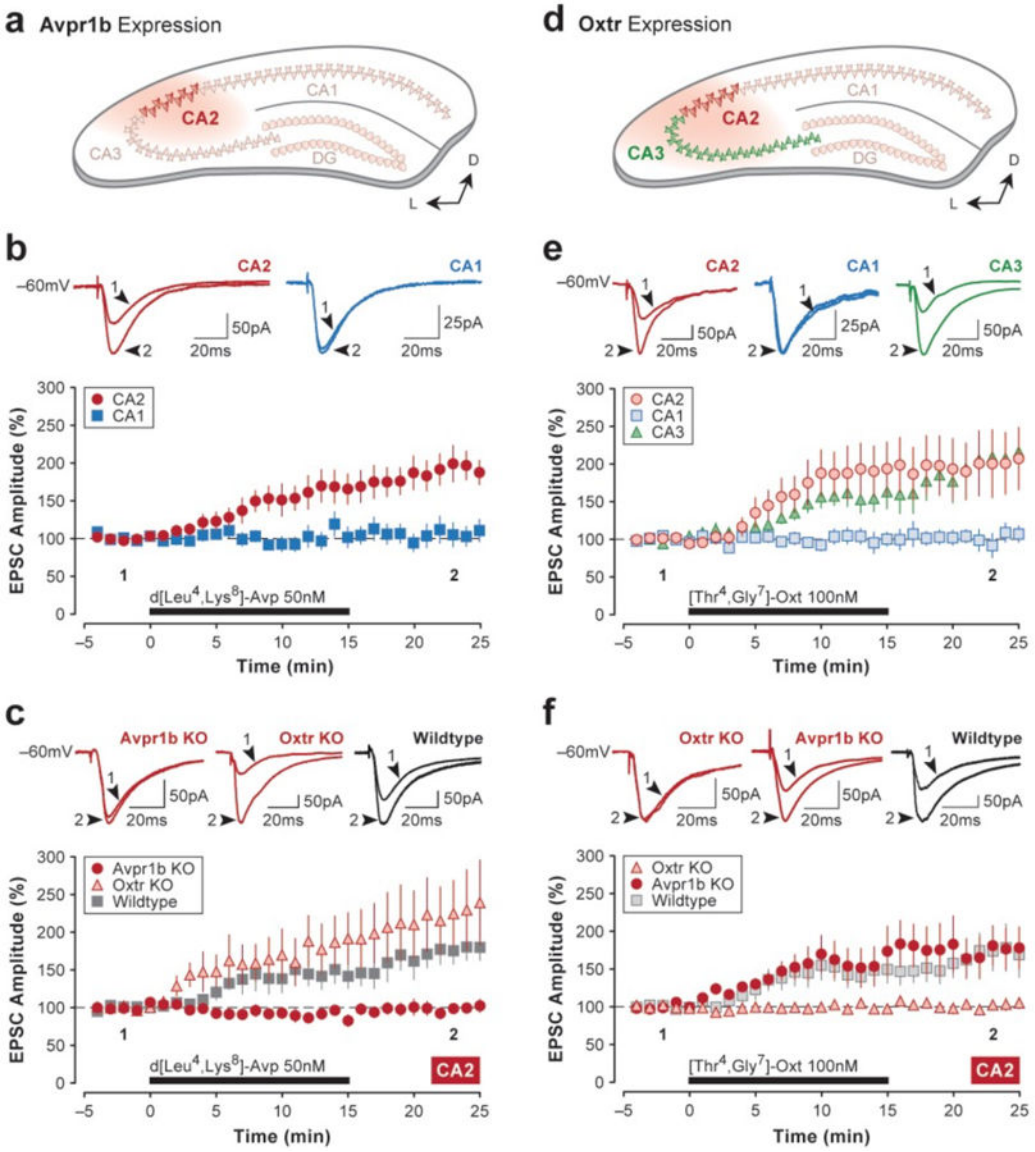

Figure 2.

Synaptic potentiation is induced by Avpr1b and Oxtr agonists in slices of rat and mouse hippocampus. **(a)** Reflecting the enrichment of Avpr1b in CA2, **(b)** 50 nM d[Leu^4,Lys^8]-Avp, a selective Avpr1b agonist, induced potentiation of EPSCs recorded in rat CA2 (red circles; n=9) but not in CA1 (blue squares; n=6). The duration of drug application is indicated by the black bar in this and subsequent figures. Representative synaptic currents from time points before (1) and after (2) drug application are shown above the averaged results for areas CA2 and CA1. **(c)** Similar effects were observed in CA2 neurons in slices from WT (grey squares; n=6) and Oxtr KO (pink triangles; n=4) mice, but not in slices from Avpr1b KO mice (red circles; n=7). Representative currents are shown above the group data at the time points indicated by the numbers. Similar results were observed with 100 nM of the Oxtr agonist [Thr^4,Gly^7]-Oxt **(d–f)**. The high levels of oxytocin receptor binding in areas CA2 and CA3 are depicted in the schematic diagram shown in **(d)**, and **(e)** synaptic

potentiation induced with the Oxtr agonist is observed in CA2 (pink circles; n=11) and CA3 (green triangles; n=7), but not CA1 (blue squares; n=8). Conventions in **(e)** and **(f)** are the same as in **(b)** and **(c)**. **(f)** In addition, potentiation induced with [Thr^4,Gly^7]-Oxt was also observed in slices from WT mice (grey squares; n=6) and Avpr1b KO mice (red circles; n=11), but not in slices from Oxtr KO mice (pink triangles; n=7).

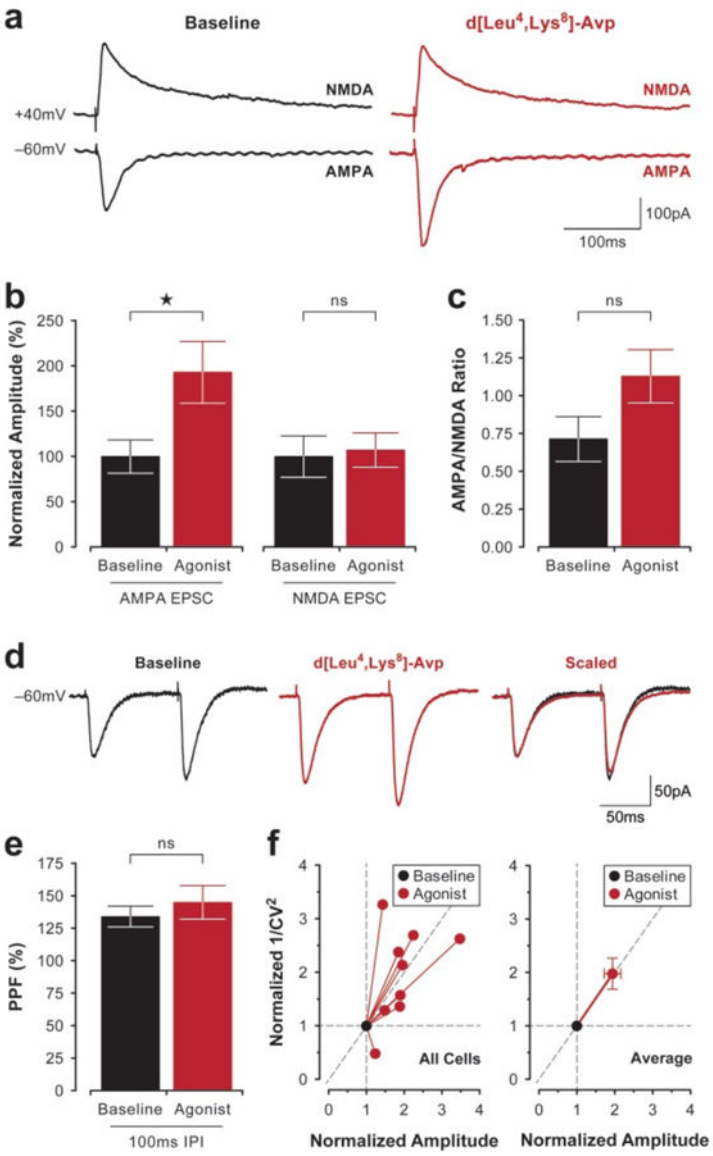

Figure 3.

The potentiation of EPSCs in area CA2 induced by d[Leu^4,Lys^8]-Avp is mediated by a postsynaptic change in AMPA receptor function. AMPA- and NMDA-mediated currents were recorded before and after bath application of 50nM d[Leu^4,Lys^8]-Avp and synaptic stimulation for 15 min. Sample currents in **(a)** show that the AMPA-mediated EPSC is enhanced following application of the agonist. **(b)** This is also reflected in the group data by a significant increase in the AMPA-mediated EPSC (star, P<0.01), but not in the NMDA-mediated EPSC (ns, non-significant; n=12 each). **(c)** The AMPA/NMDA ratio was not significantly different. **(d,e)** The Avp1b agonist, d[Leu^4,Lys^8]-Avp, induces no significant change in paired-pulse facilitation. Pairs of stimulation pulses were delivered to the SC input to CA2 separated by an inter-pulse interval of 100 msec. Although 50nM d[Leu^4,Lys^8]-Avp enhanced the amplitude of synaptic currents in CA2, there was no change in paired-pulse facilitation; scaled example currents from a representative experiment are shown in **(d)** and

group data are shown in **(e)**; n=6 each. **(f)** In addition, responses-to-response variability in the amplitude of EPSCs was reduced following application of the Avpr1b agonist as indicated by an increase in the coefficient of variation ($1/CV^2$) (n=9 each).

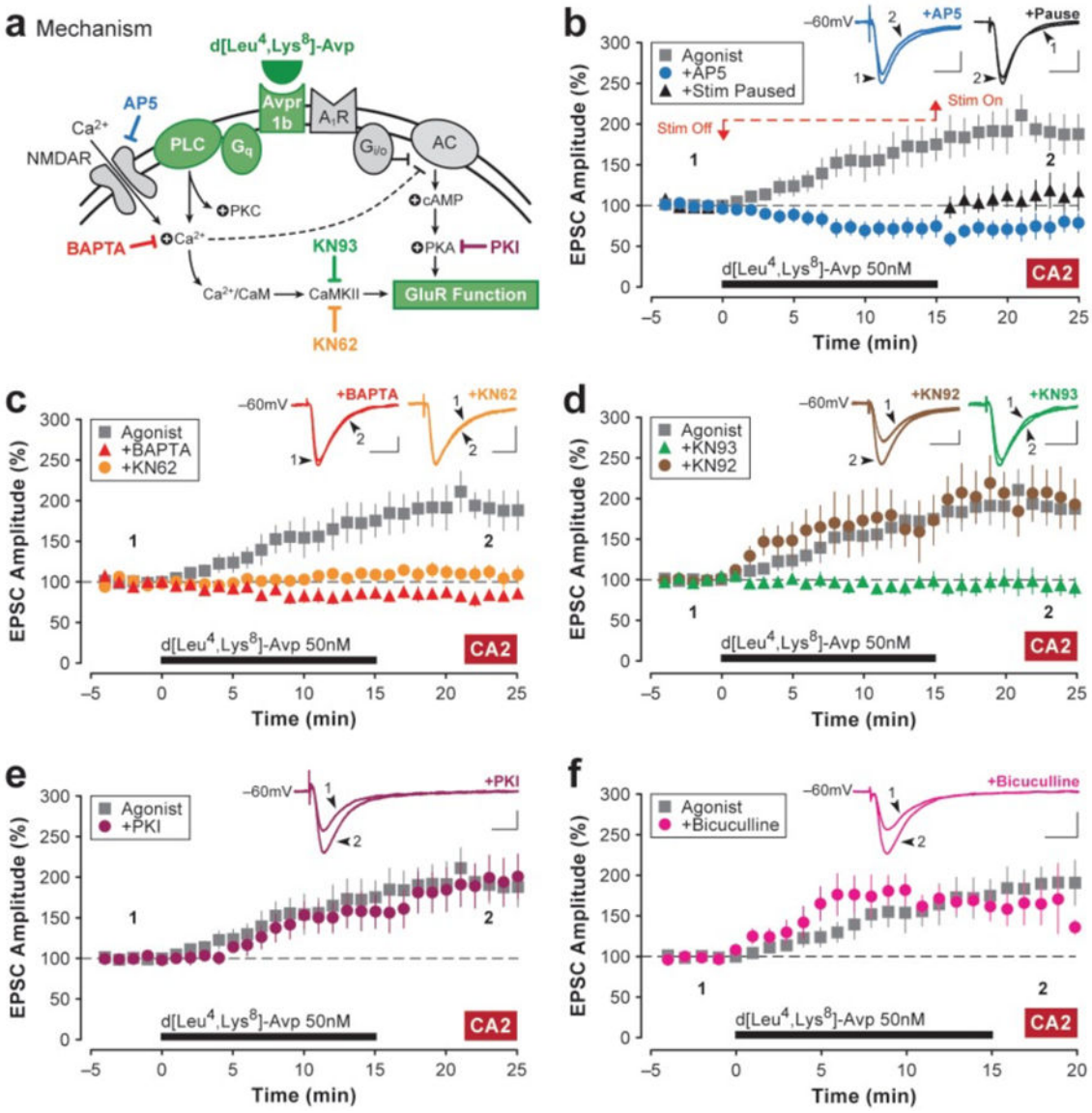

Figure 4.

Avpr1b agonist-induced synaptic potentiation in CA2 is calcium dependent. **(a)** Proposed mechanism of Avpr1b-induced potentiation based on the idea that Avpr1b is coupled to Gq proteins and phospholipase C-dependent calcium increases. This mechanism is in contrast to the potentiation induced with antagonists of the CA2-enriched A1 adenosine receptor, which acts through a PKA-dependent pathway. **(b)** Avpr1b agonist-induced potentiation is blocked by application of 50 μM AP5, an inhibitor of NMDA receptors (blue circles; n=9) or by temporarily pausing stimulation during drug application (black triangles; n=7), indicating that synaptic glutamate release and NMDA receptors are required for the potentiation. For reference in all panels, the results of application of d[Leu^4,Lys^8]-Avp is indicated by the grey squares (n=9). **(c)** Similarly,loading cells with 15 mM BAPTA, a high affinity calcium chelator, blocked the potentiation induced by 50 nM d[Leu^4,Lys^8]-Avp (red triangles; n=5), indicating that this potentiation is Ca^{2+} dependent. In addition, 10 μM KN-62 (orange

circles; n=7) and KN-93 (**d**; green triangles; n=6), inhibitors of CaMKII, included in the recording pipette also inhibited the potentiation. However, KN-92, an inactive analogue of KN-93 (brown circles; n=7), 20 μM PKI, an inhibitor of PKA, (magenta circles; n=8, **e**), or bicuculline, an inhibitor of $GABA_A$ receptors (pink circles; n=7, **f**), failed to block the potentiation. Representative traces are shown as insets at the times indicated by 1 and 2 (calibration bars: 50 pA, 20 ms).