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Tonic Regulation of GABAergic Synaptic Activity on Vasopressin Neurones by Cannabinoids

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Abstract

Synaptic activity in magnocellular neurosecretory neurones is influenced by the retrograde (*i.e.*, somatodendritic) release of vasopressin, oxytocin and cannabinoids (CBs). For oxytocin neurones, oxytocin exerts constitutive effects on presynaptic activity through its ability to release CBs postsynaptically. In the present study we examined evoked and spontaneous inhibitory postsynaptic currents (IPSCs) in identified vasopressin (VP) neurones in coronal slices from virgin rats to determine: 1) the extent to which CBs may also tonically modulate VP synaptic activity; and 2) to determine if depolarization induced suppression of inhibition was present in VP neurones, and if so, whether it was mediated by VP or CBs. The CB1 antagonists AM251 (1 μM) and SR14171 (1 μM) consistently increased the frequency of spontaneous IPSCs (sIPSCs) in VP neurones without affecting their amplitude, suggesting a tonic CB presence. This effect on frequency was independent of action potential activity, and blocked by chelating intracellular calcium with10 mM EGTA. AM251 also increased the amplitude of evoked IPSCs (eIPSCs) and decreased the paired-pulse ratio (PPR) in VP neurones- effects that were completely blocked with even low (1 mM EGTA) internal calcium chelation. Bouts of evoked firing of VP neurones consistently suppressed sIPSCs, but had no effect on eIPSCs or the PPR. This depolarization-induced suppression of IPSCs was reduced by AM251, and was totally blocked by 10 μM of the mixed vasopressin/oxytocin antagonist, Manning Compound. We then tested the effect of vasopressin on IPSCs while blocking CB1 receptors. Vasopressin (10-100 nM) inhibited sIPSC frequency, but had no effect on sIPSC or eIPSC amplitudes, or on the PPR, in the presence of AM251. Together these results suggest a tonic, presynaptic inhibitory modulation of IPSCs in VP neurones by CBs that is largely dependent on postsynaptic calcium, and an inhibitory effect of VP on IPSCs that is independent of CB release.

Keywords

supraoptic; paraventricular; GABA; vasopressin; oxytocin

Introduction

The inhibitory neurotransmitter GABA accounts for 40-50% of all synapses impinging on hypothalamic magnocellular neurosecretory cells (MNCs) in the supraoptic (SON) and paraventricular nucleus (PVN) (1-3). GABAergic transmission mediates the rapid inhibition of vasopressin (VP) neurones in the SON following acute hypertension (4, 5) and modulates oxytocin (OT) neurone bursts during lactation (6, 7). GABAergic synapses undergo significant morphological (8) and physiological plasticity during pregnancy and lactation

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(9-11), and dehydration (12). GABAergic inputs to the SON and PVN arise from several extrinsic regions, including the bed nucleus of the stria terminalis, the organum vasculosum of the lamina terminalis, the median preoptic nucleus, the subfornical organ, diagonal band of Broca, and the suprachiasmatic nucleus, whereas other inputs arise locally, from perinuclear regions of the SON (13, 14, for review) and the interneurones, or immediately adjacent neurones, of the PVN (15-17).

Although VP and OT neurones may be contacted by a similar number of GABA-containing synaptic terminals (2, 3), GABAergic activity is thought to be differentially regulated on the two cell types. In male rats, Baimoukhametova *et al.* (18) found that GABA-mediated, inhibitory postsynaptic currents (IPSCs) exhibited use-dependent plasticity, which in VP neurones took the form of synaptic depression. In contrast, in OT neurones, IPSCs showed synaptic facilitation. Synaptic depression was highly sensitive to dopamine receptor regulation, whereas facilitation was not. IPSC facilitation in OT neurones is also regulated by the constitutive release of OT and cannabinoids (CBs), the current hypothesis being that OT receptor activation on OT neurones releases CBs, which in turn act on CB1 presynaptic receptors (19)- a similar phenomenon was originally described for the retrograde regulation of excitatory postsynaptic currents in the SON (20). Examining both male and female rats, Oliet *et al.* (19) found that evoked IPSCs in VP neurones were insensitive to CB1 or VP/OT receptor blockades, suggesting constitutive CB release targeted on OT neurones.

In rat hypothalamic slices from female Sprague-Dawley rats (21), the frequency of IPSCs (and mIPSCs) in hypothalamic slices is several-fold greater in OT when compared with VP neurones (21). Given that GABAergic innervation may be roughly similar in the two cell types (2, 3), and that presynaptic spiking activity contributes little to the distribution of IPSCs in the coronal slice (9, 21, 22), we hypothesized IPSCs in VP neurones may be tonically suppressed by constitutive factors. Here we report that, in contrast to what has been reported in previous studies (18, 19), CB1 receptors can also mediate tonic suppression of spontaneous IPSCs on VP neurones *via* a presynaptic mechanism. An abstract of this work has been previously reported (23).

Materials and Methods

Animals and slice preparation

Coronal slices (250 μM) containing the supraoptic nuclei (SON) of hypothalamus were prepared from random cycling, virgin female adult rats (150-250g; Sprague Dawley, Harlan Laboratories, Indianapolis, IN). The rats were deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and perfused transcardially with ice cold, low-Na^+ (NaCl was replaced by an equiosmolar amount of sucrose) artificial cerebrospinal fluid (ACSF), which had been oxygenated with 95% O_2 and 5% CO_2. The brain was then rapidly removed from the skull, blocked in the coronal plane, glued to the stage of a vibrating slicer (VT1000s, Leica) and cut at a thickness of 250 μm into the same sucrose-ACSF slush. Slices were incubated in normal ACSF oxygenated continuously at 32-34°C for 1 h, then maintained at room temperature until transfer to a recording chamber. The ACSF contained (in mM): 125 NaCl, 2.5 KCl, 2 $CaCl_2$, 1.0 $MgSO_4$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 0.45 ascorbic acid, and 20 D-glucose (pH = 7.4; ~290 mOsm/kg). The recording chamber was continuously perfused with oxygenated ACSF at ~2ml/min at 32-34°C. Animal procedures were performed under protocols approved by the Institutional Animal Care and Use Committee at University of Tennessee.

Electrophysiological recordings

Patch pipettes (3-5 MΩ) were prepared from thin-walled borosilicate capillary glass (o.d.=1.5mm, i.d.=1.17mm, Warner Instrument Corp.) using a horizontal micropipette puller (P-80, Sutter Instruments Co.). Most experiments were done with a K-gluconate based pipette internal solution containing (in mM): 140 K-gluconate, 10 KCl, 10 Hepes, 4 Mg-ATP, 0.3 Na-GTP, 3.5 phosphocreatine, 0.2 EGTA. The pH of the pipette solution was adjusted to 7.3 with 1 M KOH, and osmolarity was adjusted to ~285 mosmol/kg. Where noted, a CsCl based internal solution also was used to enhance GABA-mediated currents, containing (mM): 120 CsCl, 30 Hepes, 0.2 EGTA, 2 $MgCl_2$, 1.0 $CaCl_2$ and 4.0 Mg-ATP (Li *et al*., 2007). Cesium hydroxide was used to adjust pH to 7.3 and osmolarity was adjusted to 295 mosmol/kg. For CsCl based recordings where extracellular stimulation was used, the Na^+ channel blocker QX-314 (1 mM) (Sigma, St. Louis, MO, USA) was added to the internal solution in order to block antidromic action currents. Biocytin (0.1%, Sigma) was added to internal solutions in order to label neurones for immunochemical identification.

Whole-cell recordings were obtained with an Axopatch 200B(Axon Instruments, Foster City, CA, USA) amplifier. The magnocellular neurosecretory cells (MNCs) in SON were visually identified on a BX50WI upright microscope (Olympus Optical, Tokyo, Japan) using infrared light, differential interference contrast (IR-DIC) and a CCD camera (Sensicam, TILL Photonics). The membrane currents were recorded without series resistance compensation, filtered at 2 kHz and digitized at 20kHz with a Digidata interface (1320A; Axon Instruments) in conjunction with pClamp 9 software (Axon instruments), and stored for off-line analysis. Voltage outputs were filtered at 10 kHz and digitized at 20 kHz. A 5mV, 10 msec depolarizing pulse was applied regularly throughout each experiment, and the steady-state current and decay rate (τ) of the capacitance transient were monitored as measures of input resistance and series resistance, respectively. The cells were excluded from data analysis if more than 20% change in series resistance occurred during the experiment. Spontaneous (sIPSCs) and evoked (eIPSCs) inhibitory postsynaptic currents were recorded at a holding potential of −40 mV (K-gluconate based internal solution) or −70mV (CsCl based internal solution) respectively. All the experiments were conducted with DL-2-amino-5-phosphonopentanoic acid (AP5; 40 μM) and 6,7-dinitroquinoxaline-2,3(1*h*,*4h*)-dione (DNQX; 10 μM) to block any glutamatergic synaptic events. Miniature IPSCs (mIPSCs) were recorded in presence of tetrodotoxin (TTX; 0.5 μM, Sigma). No corrections were made for the pipette liquid junction potentials (~10 mV in gluconate internal, ~3 mV in CsCl internal). In our lab, IPSCs are virtually all blocked with gabazine or picrotoxin, and therefore mediated by Cl^- currents through $GABA_A$ receptors (21).

In order to evoke eIPSCs, extracellular electrical stimuli were applied to a region just dorsomedial to the SON, close to the optic tract, through a concentric bipolar stimulating electrode connected to a stimulus isolation unit (Winston Electronics, CA). Pairs of square pulses (100 μs duration, 50 ms interpulse interval) with an intensity that generated 50% of the maximum IPSC amplitude were used to provide a stable response over 20-30 trials (15 s intertrial interval). The paired-pulse ratio (PPR) was quantified by the ratio of the average amplitude of the second eIPSC to the average of the first eIPSC. To further determine retrograde effects on ISPCs, a sustained depolarizing current (1s duration) was injected into recorded SON neurones in current-clamp mode before switching to voltage clamp for IPSC assessment. Four consecutive injected currents (at intervals of 10s) were used, with an intensity that yielded action potentials at 45-55 Hz, a frequency known to trigger local peptide release *(e.g*., 24, 25),

Data analysis

The sIPSCs were automatically detected and analyzed using MiniAnalysis software (v 6.0, Synaptosoft, Decatur, GA). The program selected all events with a peak detecting threshold at five times the root mean square (RMS) noise of the baseline and had typical spontaneous IPSCs kinetics. Accurate detection was verified by subsequent visual inspection and editing. IPSCs were analyzed with Clampfit 9.0 software (Axon instruments). The amplitude of the evoked synaptic current was calculated from the baseline before the first evoked response to the peak of the current.

The nonparametric Wilcoxon's Signed-Rank paired test was used to determine the statistical significance of within-cell drug effects. Group differences were compared with the nonparametric Mann-Whitney *U* test. Probability values of $p<0.05$ were considered to be statistically significant. All data are presented as means + S.E.M. Tests were run with Statview 5.0 (Adept Scientific Inc, Bethesda, MD, USA).

Immunocytochemistry

We identified OT and VP neurones with protocols similar to those published (e.g., 26, 27). Slices were fixed in 4% paraformaldehyde and 0.2% picric acid dissolved in 0.15M phosphate buffered (PH 7.4), then rinsed in phosphate-buffered saline (PBS: 0.01 M Na phosphate buffer, 0.15 M NaCl) containing 0.5% Triton X-100 (TX) and incubated in primary antibodies for 2 to 3 days at 4°C. VP neurones were identified by a polyclonal antibody (VP-NP, provided by Alan Robinson, retired) raised in rabbit against VP-neurophysin and used at a 1:20,000 dilution. OT neurones were identified by a monoclonal antibody (PS36 or PS 38, provided by Harold Gainer, NIH) raised in mouse against OT-neurophysin and used at a 1:5000 dilution. All primary antibodies were dissolved in PBS-TX containing 0.04% sodium azide. After incubation in primary antibodies, the slices were rinsed in three changes of PBS-TX, then incubated in a cocktail of secondary antibodies and avidin-7-amino-4-methylcoumarin-3-acetic acid (avidin-AMCA, Vector labs, Burlingame, CA) 4-6 h at room temperature. The secondary antibodies were fluorescein isothiocyanate (FITC)-conjugated or Alex Fluor 488-conjugated goat anti-rabbit IgG, and Alexa Fluor 594-conjugated goat anti-mouse IgG (Invitrogen, Eugene, OR). Avidin-AMCA was used to label the biocytin-filled neurones. Photographs were captured using a digital camera (Sensyscam, Photometrics) with IPLabs software (Scanalytics, Fairfax, VA). Neurones were considered as either OT or VP types only if positive staining of one antibody was accompanied by a negative reaction with the other. A small minority of neurones was labelled with both antibodies, which has been reported previously (21). These cells were not used for analysis in the present study.

Drugs

AM251 and SR141716A were purchased from Tocris Bioscience (Ellesville, MO). Arg-vasopressin and Manning compound were purchased from Bachem Americas (Torrance, CA). Unless otherwise stated, all other reagents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO).

Results

CB1 receptor blockade increases IPSC frequency and alters release probability in VP neurones

As previous studies have suggested a role for constitutively released CBs in regulating synaptic activity in MNCs (19, 20), we tested the CB1 antagonist AM251 (1 μM) on sIPSCs in VP and OT neurones. AM251 consistently increased the frequency of sIPSCs in VP ~2-fold, with no change in sIPSC amplitude (Fig. 1). We also tested a different CB1 antagonist,

SR141716A, to be certain these effects were not specific to AM251. Although somewhat less pronounced, SR141716A (1 μM) also statistically increased the frequency of sIPSCs in VP neurones (Fig. 1). Unfortunately, sIPSCs in OT neurones, but not in VP neurones, showed significant rundown over the extended time periods needed for testing periods (data not shown), so we chose not to make direct comparisons between the two cell types nor include OT neurones in the other analyses.

The increase in sIPSC frequency in VP neurones in response to AM251 suggested a presynaptic action. We examined AM251's effect on the paired-pulse ratio (PPR) using evoked IPSCs (PPR = eIPSC2/eIPSC1) (19). As shown in Fig. 2, AM251 (1 μM) significantly decreased the PPR, and simultaneously increased the amplitude of eIPSC1, consistent with an increase in the probability of transmitter release. Because our results differed from the only previous report examining identified ISPCs in VP neurones with CB1 antagonists (19), we tested some VP neurones with a 140 mM CsCl based internal solution similar to one used in that study and found that AM251 (1 μM) moderately increased sIPSC frequency (2.75 ± 0.5 *vs*. 3.14 ± 0.60 Hz; $p \leq 0.005$, n= 13) and decreased the PPR (1.42 ± 0.14 *vs*. 1.19 ± 0. 14; $p \leq 0.033$; n = 13). The tonic CB effect on the PPR was calcium-dependent, as increasing EGTA from 0.2 to 1 mM in the pipette solution prevented significant AM251 effects on PPR (1.31 ± 0.12 vs. 1.25 ± 0.12; $p \leq 0.56$; n = 23). AM251 (1 μM) did continue to a significant increase in sIPSC frequency with the higher internal calcium chelation (3.84 + 0.80 Hz *vs*. 4.64 + 0.85 Hz; $p < 0.006$; n = 26).However, this persistence of AM251's effect on sIPCSs likely reflected incomplete calcium buffering. In another seven neurones, we raised EGTA to 10 mM in the pipette using the K gluconate internal solution, and completely blocked AM251's effect on sIPSCs (1.44 ± 0.43 Hz vs. 1.24 ± 0.27; $p \leq 0.86$; n = 7). Together, these results suggest that calcium-dependent constitutive release of CBs provides presynaptic, tonic inhibition of sIPSCs .

Although most studies suggest that sIPCS in the SON from coronal slices are largely mIPSCs and thus spike-independent (e.g., 9, 21), we tested the effect of AM251 in TTX to insure the increase was not due to exciting silent GABAergic neurones. Using the CsCl internal solution, AM251 (1 μM) continued to increase mIPSCs in the presence of 0.5 μM TTX (4.16 ± 0.82 Hz *vs*. 6.55 ± 1.20; $p \leq 0.02$; n = 15).

Spike activity in VP neurones further decreases IPSC frequency

To further determine whether a single VP neurone's spiking activity can contribute to local, retrograde synaptic modulation of IPSCs impinging on that neurone, we used a depolarization pulse protocol (DPP) similar to that previously employed in the SON (20, 24). sIPSCs were recorded in voltage clamp before and after inducing firing bouts in the recorded neurone using DPP in current clamp. As shown in Fig. 3, DPP resulted in suppressed sIPSC frequency in VP neurones without affecting sIPSC amplitude. This effect was long lasting, recovering only to 67.8 + 6.8% of control levels after 15-20 mins. To determine whether this depolarization-induced suppression of inhibition, or DSI, was associated with a change in release probability, we also examined the amplitude of eIPSCs and the PPR before and after DPP. Despite lowering sIPSC frequency, DPP produced no significant change in the PPR or in the amplitude of eIPSC1 (Fig. 3), suggesting that DPP's strong effect on frequency is likely not mediated by altering a calcium-dependent presynaptic mechanism that might be typically associated with PPR and its plasticity (28).

Since both CBs and VP may be released locally by VP neurones, we first tested AM251's effect on DSI. While AM251 did not totally prevent DSI in VP neurones (Fig. 4), the magnitude of DSI was significantly less in the presence of AM251 (14.5 ± 5.7%; n = 14) than it was without drug (34.1 ± 5.8 %; n = 12) ($p \leq 0.02$ comparing these two percentages), suggesting some contribution of CB1 receptor activation to DSI. However, recovery of DSI

remained slow in AM251, as it was in control conditions. As with DPP alone, DPP in the presence of AM251 produced no effect on PPR or eIPSC amplitude.

We repeated the DPP experiment in the presence of VP/OT receptor blocker, Manning Compound (MC) (10 μM). As shown in Fig. 4, in contrast to AM251, MC completely blocked DSI. This suggests at least partially independent involvement of CBs and VP in controlling sIPSCs.

VP Inhibits sIPSCs Independently from CBs

Because the constitutive effects of CBs on excitatory (20) and inhibitory (19) synaptic transmission in the SON were previously related to peptide release, we tested whether or not VP could exert actions independent of CBs on IPSCs. In the presence of 1 μM AM251, VP (10 nM) reversibly inhibited sIPSC frequency without altering amplitude. The effects of VP were transient, and fully recovered with 15-20 min washing (115.3 ± 28.2% of control). Like the effects of DPP, VP did not reduce eIPSC amplitude, nor alter PPR (Fig. 5), suggesting that the effect of VP on sIPSC frequency in VP neurones likely was not mediated by calcium-dependent mechanism at the presynaptic terminal. Increasing VP to 100 nM produced a similar, but not greater, effect on IPSC frequency as 10 nM ($n = 5$; $p \leq 0.04$).

Discussion

The present results demonstrate that inhibitory synaptic transmission in identified VP neurones from female virgin rats (randomly cycling) is presynaptically modulated by the constitutive release of CBs via CB1 receptors. These results stand in contrast to a previous study suggesting that only in OT neurones were GABAergic inputs under CB tonic control (19). While it may be hard to reconcile these conflicting results, there are important differences in the studies. Oliet *et al.* (19) examined evoked IPSCs and PPR in younger (3-8 weeks) animals of both sexes, whereas we used adult female virgin rats as in our past studies of excitatory transmission (29, 30), and studied both evoked and spontaneous release. We chose a gluconate solution for the majority of this study because the high internal Cl^- concentration created large IPSCs that could sometimes activate large inward currents-possibly because remote synapses not under good voltage control produced large depolarizations sufficient to activate voltage gated Ca^{++} channels. Nevertheless, we did test a group of VP neurones with a CsCl based internal similar to that of Oliet *et al.* (19), and found the same effects of AM251 as with the gluconate internal. In addition we determined that the excitatory effect of AM251 is not due to increasing the spiking of low firing or silent presynaptic GABAergic neurones, since it persisted in TTX. Due to rundown of sIPSCs in OT neurones, we did not compare these two cell types. However, we previously found that CB1 blockade reduced PPR in OT neurones using the CsCl internal solution (23), similar to the effect reported by Oliet *et al.* (19).

We should also note that Oliet *et al*. (19) used a higher concentration of AM251 (5 μM). We chose a concentration of 1 μM for AM251 and SR141 to avoid non-specific effects, since AM251's reported affinity is in the low nanomolar (even sub-nanomolar) range for CB1 receptors (31-33)- several orders of magnitude below 1 μM. Concentrations above 1 μM may competitively react with adenosine receptors (34) and AM251's K_d for the CB2 receptor is ~100 nM (33). Furthermore the initial work reporting constitutive CB activity in brain used low doses (0.3 μM; *ref. 35*; 2 μM; *ref.* 36). And finally, previous work in SON neurones showed that 1 μM AM251 completely blocked CB effects after CB uptake blockade (37), blocked the effects of two exogenously added, naturally occurring CBs (37) and blocked the effects of the specific agonist, WIN 55,212-2 (20; 37). 1 μM AM251 also completely blocked the effect of oxytocin-induced release of CBs on evoked EPSCs (20).

A caution regarding the interpretation of the effects of CB antagonists is that AM251 and SR141716A can both act as inverse agonists at the CB1 receptor (38, for review). Indeed, estimates indicate that ~85% of antagonists to G-protein coupled receptors, in general, may behave as inverse agonists (39). This type of action implies that the receptor itself is constitutively active and thus the effects of the antagonists don't always imply constitutive CB release. In the present study, chelating $[Ca^{++}]_i$ with 10 mM EGTA blocked AM251's effects on the PPR and sIPSCs, suggesting that constitutive release of CB from that individual neuroneprovided tonic inhibition. The effects on PPR and IPSC frequency both point to a presynaptic location of the CB1 receptor, consistent with previous anatomical reports (20), but the IPSC frequency and pair-pulsed facilitation/inhibition may be differentially controlled by CBs, or perhaps differentially sensitive to CB concentration. This is supported by the fact that moderate chelation of calcium with 1 mM blocked the PPR effects of AM251, but only stronger chelation (10 mM EGTA) prevented the increased sIPSC frequency we commonly observed in response to AM251.

We found important differences between the constitutive CB effects and those attributed to retrograde release induced by spiking of the recorded VP neurone. Constitutive CB release, as measured by the effects of CB1 antagonists, suppressed IPSC frequency and reduced the probability of GABA release; we interpret both as presynaptic effects, consistent with previous data on the terminal location of CB1 receptors in the SON and the inhibitory effects of CBs on EPSCs (20) and IPSCs (19) in both VP and OT SON neurones. However when inducing the recorded neurone to spike, presumably further increasing the release of CBs (as well as VP and probably other factors) by increasing intracellular calcium, we consistently found suppression of sIPSC frequency but no changes in eIPSC amplitude, or in the PPR. Thus although perhaps presynaptically mediated, the DSI-induced changes would appear not to invoke the same type of calcium-dependent presynaptic inhibition as constitutive CB release, since neither the PPR nor eISPC amplitude was affected. Previous studies examining retrograde effects of VP or OT on IPSCs in the SON or PVN did not report testing for DSI (18, 19). Interestingly, the DSI effect appeared longer lasting than inhibitory retrograde effects of evoked EPSCs reported by Kombian *et al.* (24). The reason for this difference is not obvious since the effects of exogenous VP were reversible over the same time frame (15-20 mins), whereas when blocking CB1 receptors DSI still produced a persistent effect. This suggests a prolonged release of the intrinsically released VP following the DPP protocol, or some other factor that is dependent upon VP release, since DSI was blocked with MC. A prolonged action of retrograde messengers is not unprecedented. In a study of retrograde opiate (dynorphin) inhibition in VP neurones, Iremonger and Bains (40) found that DPP and opiate agonists produced a persistent depression of EPSC frequency. These authors concluded through a number of experiments that dynorphin released from VP neurones suppressed presynaptic glutamate release machinery downstream of presynaptic calcium. While our basic DSI effect is similar since it was prolonged, and did not affect IPSC amplitude or PPR, if VP per se is the presynaptic agent, we are unable to explain why exogenous VP does not itself produce a prolonged effect. A simple explanation may be the small doses (10-100 nM) we used-DPP may cause larger increases in extracellular VP near the point of action, and these lower doses could be more easily washed out. The DPP protocol may also invoke some prolonged postsynaptic changes governing release- perhaps a prolonged increase in intracellular calcium that sustain the release of the retrograde messenger.

A difference in the effects of DSI and constitutive release CB in VP neurones may relate to the involvement of factors co-released with CBs. Thus the VP/OT receptor blocker MC completely blocked, whereas AM251 only reduced, DSI in VP neurones. This is in contrast to effects on excitatory synapses reported by Hirasawa *et al.* (20), who found a suppression of eEPSC amplitude following postsynaptic depolarization that was equally blocked by

AM251 or MC. While this depolarization also reduced mEPSC frequency, the effects of the two antagonists on this response were untested in that study. Interestingly, when blocking CB1 receptors, Hirasawa *et al.* (20) found that OT (1 μM) actually *increased* the frequency of sEPSCs. In addition our results for VP neurones are surprising since in identified OT neurones, the constitutive, suppressive effects reported for OT on eIPSCs appeared entirely due to this peptide's ability to promote CB release and in turn affect presynaptic CB1 receptors (19), similar to the conclusion of Hirasawa *et al.* (20) regarding retrograde effects on EPSCs. We further examined the direct effects of VP while blocking CB1 receptors, and found a small inhibition of sIPSC frequency, but again, no change in PPR or in eIPSC amplitude. Thus while OT autoreceptor activation leads to CB release, which in turn activates presynaptic CB1 receptors on terminals releasing glutamate or GABA, and seems generally inhibitory, we found that locally released VP appears to have effects on presynaptic sIPSCs that are at least partially independent from CB1 involvement. While VP (*e.g*., V1a) receptors appear to be present on VP neurones, there is little corroborative evidence for their presynaptic location in the SON (41, 42). In the SON, VP was found to suppress eEPSCs and glutamate-induced currents in VP neurones *via* a postsynaptic mechanism, and enhance them on OT neurones *via* cross-talk with OT receptors (41). In contrast, in the nucleus of the solitary tract, presynaptic V1a receptors are known to inhibit glutamatergic synaptic transmission (43). Since anatomical studies do not at present suggest a presynaptic location for VP receptors in the SON, the actions of MC and VP we observed on sIPSC frequency could be related to VP's ability to act on VP autoreceptors and release yet another retrograde factor, much in the same way that OT is thought to promote CB release.

It should not be surprising to find a suppression of sIPSC frequency due to VP, or to DSI, without observing effects on evoked release. In many parts of the nervous system, spontaneous transmitter release provides an independent signaling pathway from evoked release, different in its postsynaptic effects, and for the present discussion, in its modulation. Kavalali *et al.* (44) recently reviewed the data in support of this independence. Changes in evoked *vs.* spontaneous release need not occur together, and their signs may even be opposing, or when similar, greatly different in scale. There are many mechanisms that can underlie these differences, but a common denominator appears to be the ability of some presynaptic modulators to target different protein pathways governing vesicle fusion. A corollary of this independence is that each type of release may, in some cases at least, use a different set of synaptic vesicles. Thus DSI, probably through VP, appears to inhibit sIPSCs through a pathway (perhaps by evoking release of another factor) at least partially different from that of CBs, and one that likely does not dramatically alter terminal calcium, as judged by the absence of PPR or eIPSC amplitude effects. A previous study likewise found no effect of VP on eIPSC amplitude, but did not examine mIPSCs or PPR (45).

In the SON, changes in spontaneous GABA release are sufficient to drive or modulate evoked activity (21), and work in the SON prior to this study revealed differences between spontaneous and evoked release, suggesting a functional role for spontaneous synaptic events. For example, high frequency afferent stimulation in the SON invokes short-term potentiation of mEPSP frequency, without any apparent change in evoked release, and this potentiation is sufficient to promote continued spike activity (46). When blocking CB1 receptors, the excitatory effect of OT on mEPCS in all SON neurones tested was accompanied by a diverse response in mEPSC amplitude, such that VP neurones more often showed a decrease in mEPSC amplitude, OT neurones an increase (20). These differences may reflect differing postsynaptic actions of VP and OT receptors (41). Reducing mIPSP frequency reduces spike train variability in the SON in both OT and VP neurones *in vitro* (21); this is significant because the frequency of spontaneous synaptic events is thought to be much higher *in vivo* (47). Even bursts of mIPSPs have been recorded in the SON, and can

occur synchronously; such bursts are capable of inducing or terminating action potential firing, depending on their timing (48). Local inhibition of GABAergic activity in the SON *in vivo* was shown to excite VP neurones in lactating female rats. (49), suggesting that spontaneous inhibitory activity can be sufficient to tonically influence firing activity. In contrast, other neurotransmitters in the SON, such as adenosine, produce consistently inhibitory effects, reducing both evoked GABA and glutamate release, and decreasing both mIPSC and mEPSC frequency (50).

The results suggest that CBs, like dynorphin and VP itself among other substances, are retrograde factors contributing to the balance of excitation and inhibition of VP neurones. However our results suggest an excitatory retrograde effect following activity, unlike the feedback inhibition of MNCs neurones that would be provided by the suppression of EPSCs previously reported for retrograde release of CBs, VP or dynorphin (20, 40). DSI similarly adjusts post-activity excitability in other neuronetypes both within (51) and outside (52) the hypothalamus- notably some of the earliest studies of retrograde CB effects in hippocampus (35, 36). Another constitutively active factor, nitric oxide, restrains VP spontaneous activity, but through the *excitation* of GABAergic transmission (53, 54). However, initiating activity in VP neurones is often followed not by inhibition but instead by a prolonged burst of activity and even long bouts of phasic activity (55-57). This suggests that the net effect of postsynaptic cell activity in VP neurones on synaptic inputs may favor excitation. In addition, short-term facilitation of excitatory synapses (including, mEPSPs) could increase the excitatory drive of MNCs (46). Perhaps DSI then contributes to the phasic, excitatory after discharges of VP neurones that while dependent on synaptic activity, maybe be initially generated through intrinsic excitatory processes, such as the depolarizing afterpotential and the consequent plateau potential that supports phasic bursting activity.

Although central CB1 receptors could be targeted by the pharmacological use of CB agonists or antagonists, a precise physiological role for CB1 receptors in the SON is unknown. Concerning hypothalamic function, CBs and CB1 receptors have been implicated in the stress response and the hypothalamic-pituitary-adrenal function, especially through the release of glucocorticoids and their hypothalamic actions on CB release (58; 59). However pain and some other stressors have been found to activate VP neurones (60; 61), and magnocellular neurones are affected by glucocorticoids (37), through complex interactions on both excitatory and inhibitory inputs (62; 63). However the argument has been made that glucocorticoids signal a rapid inhibition of magnocellular and paravocellular neuroendocrine neurones through opposing actions on inhibitory and excitatory inputs, through different mediators (NO and CBs, respectively). Thus how the apparent activation of VP neurones by stressors relates to this glucocorticoid-mediated inhibition through synaptic inputs is unknown. In addition CBs and CB1 receptors in both magnocellular and parvocellular PVN neurones have been implicated in energy balance responses *via* leptin signaling (63). Clearly, the novel constitutive and retrograde ability of CBs and VP to suppress GABAergic inputs demonstrated here needs further study in the context of these complex physiological functions.

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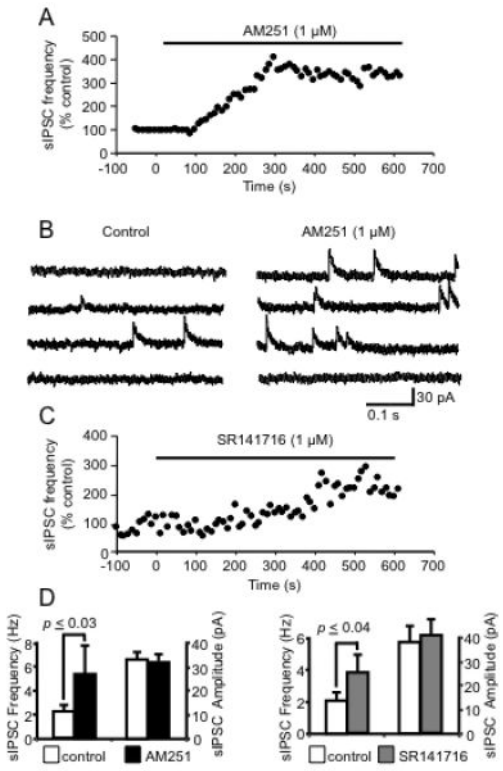

Figure 1.

CB1 receptor antagonists irreversibly increased sIPSC frequency in VP neurones without affecting amplitude. **A**, Effects of 1 μM AM251 on a single VP neurone. sIPSC frequency is averaged over 10 s. **B**, Traces of sIPSCs from the cell plotted in **A**. **C**, Effects of 1 μM SR141716 on a single VP neurone. **D**, Summary of effects of AM251 (left panel; (n = 14) and SR141716 (right panel; n = 5) on sIPSCs. Both antagonists significantly increased sIPSC frequency without affecting amplitude.

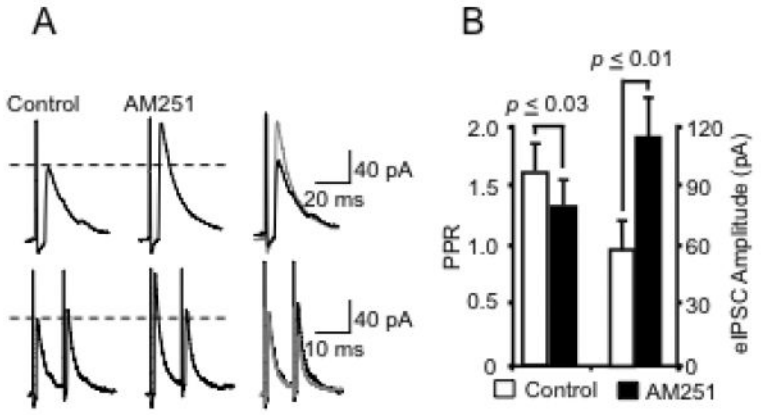

Figure 2.

The CB1 antagonist AM251 (1 μM) increased the eIPSC amplitude and reduced paired pulse facilitation (PPR) in VP neurones, suggesting probability of GABA release was increased. **A**, Averaged traces (n = 10) from a single VP neurone showing the amplitude change (upper traces) and PPR decrease (lower traces). For both panels, traces at right are overlayed, with the traces from AM251 in gray. The PPR traces at the right are scaled to the first eIPSC from controls. **B**, Summary of the effects of 1 μM AM251 on PPR and eIPSC amplitude (n = 10).

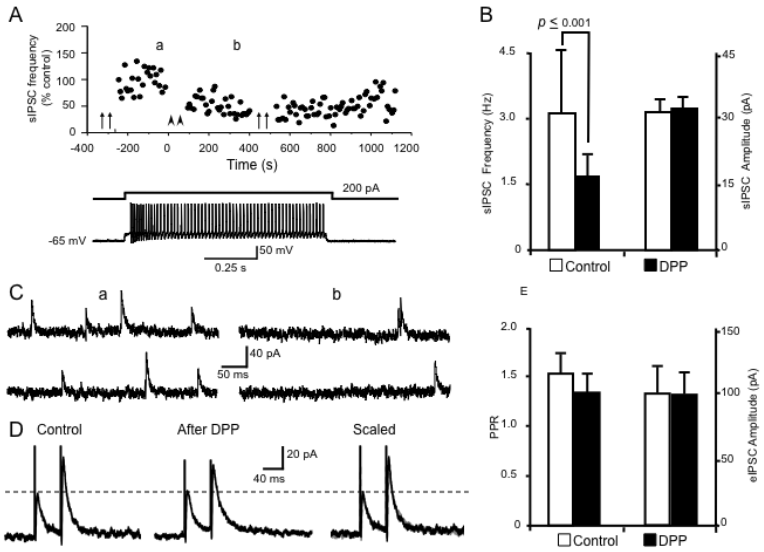

Figure 3.

Depolarization pulse protocol (DPP) reduced sIPSC frequency in VP neurones without affecting amplitude or PPR. **A**, DPP reduced sIPSC frequency in a single VP neurone. Each dot represents an average over 10 sec. The pair of arrowheads at time 0 indicates when DPP was applied (see text for details). Pairs of upward arrows indicate periods when PPR was tested, in a control period (left pair) and after DPP (right pair). The lower inset shows example of evoked spikes used in DPP. **B**, Summary of the inhibition of sIPSC frequency, but not amplitude, following DPP (n = 14). **C**, Example of sIPSCs in a control period (a) and following DPP (b). **D**, DPP failed to alter PPR in VP neurones. Averages of 10 PPR traces, with those at right overlayed (after DPP in gray) and scaled to the initial eIPSC. **E**, Summary of the effects of DPP on PPR and eIPSC amplitude (n = 9).

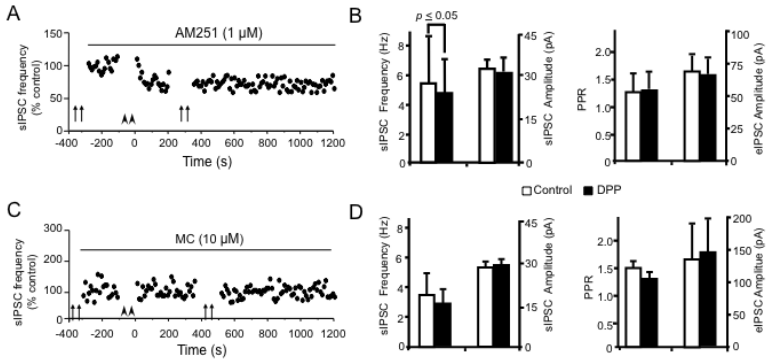

Figure 4.

A VP receptor antagonist blocked the inhibition of sIPSCs following DPP. **A**, The CB1 antagonist AM251 partially blocked DPP-induced inhibition of sIPSCs in a single neurone. Dots represent averages of 10 s. Legend as in Fig. 3A. **B**, Summary showing continued DSI in the presence of AM251, although attenuated from control (see Fig. 3), with no effect on IPSC amplitudes or PPR (n = 12). **C**, Manning Compound (MC), a VP/OT antagonist, blocks DSI in a single neurone. Legend as in **A**. **D**, Summary showing MC significantly blocked DSI with no effect on IPSC amplitude or PPR (n = 8).

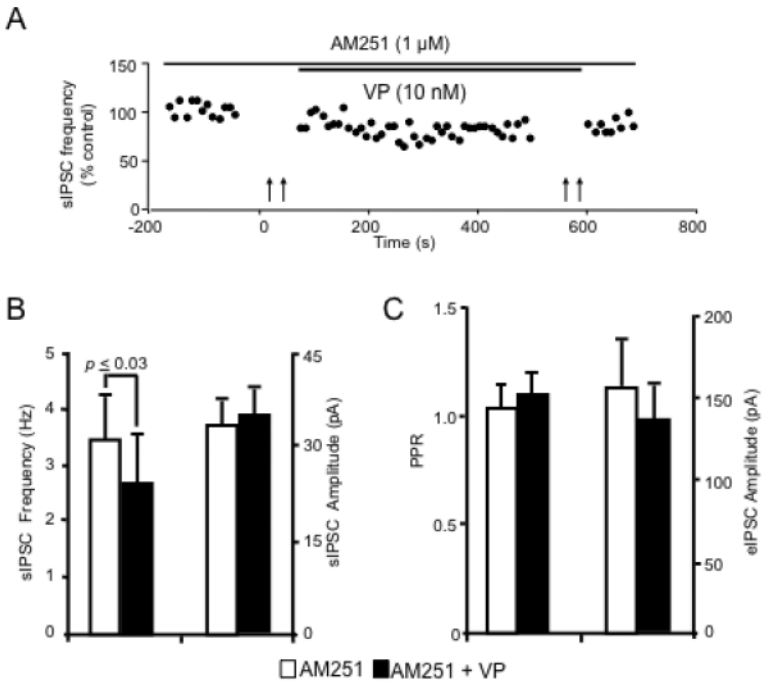

Figure 5.

VP reversibly reduced sIPSCs frequency in the presence of AM251. **A**, VP (10 nM, application indicated by thick line) decreased sIPSC frequency in a single neurone in the continued presence of 1 μM AM251 (thin line). Dots are averages of 10 s. Double arrows indicate periods when PPR was tested. **B**, Summary showing VP's inhibition of sIPSC frequency without affecting amplitude (n= 11). **C**, Summary showing VP's failure to affect PPR or eIPSC amplitude (n = 9).