# Regulation of Gonadotropin-Releasing Hormone Secretion by Cannabinoids

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## Abstract

Cannabinoids (CBs) exert untoward effects on reproduction by reducing LH secretion and suppressing gonadal function. Recent evidence suggests these effects are due primarily to hypothalamic dysfunction; however, the mechanism is obscure. Using immortalized hypothalamic GnRH neurons, we find these cells produce and secrete at least two different endocannabinoids. Following release, 2-arachidonyl monoacylglycerol and anandamide are rapidly transported into GnRH neurons and are degraded to other lipids by fatty-acid amide hydrolase. The immortalized GnRH neurons also possess CB1 and CB2 receptors that are coupled to Gi/Go proteins whose activation leads to inhibition of GnRH secretion. In perifusion experiments, CBs block pulsatile release of GnRH. When a CB receptor agonist is delivered into the third ventricle of adult female mice, estrous cycles are prolonged by at least 2 days. Although *in situ* hybridization experiments suggest either that GnRH neurons *in vivo* do not possess CB1 receptors or that they are very low, transcripts are localized in close proximity to these neurons. Inasmuch as GnRH neurons *in vivo* possess G protein receptors that are coupled to phospholipase C and increased intracellular $Ca^{2+}$, these same neurons should also be able to synthesize endocannabinoids. These lipids, in turn, could bind to CB receptors on neighboring cells and perhaps, GnRH neurons, to exert feedback control over GnRH function. This network could serve as a novel mechanism for regulating GnRH secretion where reproductive functions as diverse as the onset of puberty, timing of ovulation, duration of lactational infertility, and initiation/persistence of menopause may be affected.

### Keywords

GnRH; cannabinoids; immortalized GnRH neurons; reproduction

## INTRODUCTION

Cannabinoids (CBs) have long been known to exert potent negative effects on rodent, primate, and human reproduction (1). Chronic administration of CBs to male rodents and humans reduces sperm counts, depresses serum testosterone concentrations, and may suppress levels of LH in serum (1–3). In females, chronic CB exposure delays sexual maturation, disrupts menstrual or estrous cycles, depresses follicular maturation of the ovary, alters uterine and vaginal cytology, and may reduce contents of LH and sex steroids in blood (1,4–6). Injections of $\Delta^9$-tetrahydrocannabinol (THC) over 10 days can decrease hypothalamic GnRH concentrations in a dose-dependent manner (7). Despite these marked changes, some of the symptoms are alleviated over time due to tolerance (5) and there is rapid re-establishment of function following cessation of CB use or exogenous gonadotropin administration (1,2). By contrast, acute THC exposure inhibits pulsatile LH secretion (8) and it also blocks estrogen or estrogen/progesterone-mediated positive feedback on the LH surge in ovariectomized rats (9). Although very low doses of CBs suppress LH secretion in rodents and humans (8,10), dose-dependent effects are evident as the duration of blockade is prolonged (8). In contrast to LH, effects of CBs on FSH and prolactin secretion are more variable (1,11–15). If THC is given on the afternoon of proestrus, the LH surge and ovulation are delayed by 24 h (13,16). This blockade can be relieved through administration of exogenous gonadotropin or synthetic GnRH (8,13,14,17). In male rats administration of THC into the third ventricle depresses serum LH contents and increases concentrations of GnRH within the medial basal hypothalamus (15). By comparison, addition of the CB to cultured anterior pituitary cells exerts no effects on basal or GnRH-stimulated LH release (7,15). Hence, CB effects on LH release appear to be upstream of the pituitary. As GnRH is a major regulator of reproduction in mammals, these collective findings suggest that CBs may perturb reproduction through actions on hypothalamic GnRH neurons.

While marihuana and THC have been known to exert biological actions from some time (1), CB receptors were only cloned within the last decade (18,19). The CB1 receptor is expressed primarily within the central nervous system, whereas the CB2 receptor is found in the periphery and in immune cells. Following identification of CB receptors, investigators began to search for their endogenous ligands. These compounds are lipids and include anandamide (ANA), 2-arachidonyl monoacylglycerol (2-AMG), noladin ether, virodhamine, and *N*-arachidonyldopamine (20). Interestingly, there is some suggestion that CBs may play a role in reproduction as ANA levels in hypothalamus are low prior to puberty, increase just before puberty, and return to intermediate values immediately after vaginal opening (21). In addition, while autoradiography and *in situ* hybridization have shown that binding and expression levels of the CB1 receptor are low in hypothalamus (22), the highest levels of expression in this brain region are in the medial preoptic area and arcuate nucleus (23). Since GnRH perikarya are located in the preoptic area and these neurons send their axons through the arcuate nucleus to the median eminence to regulate gonadotropin release (24), some of the CB effects on reproduction may be mediated through the hypophysiotrophic GnRH neurons. To examine this possibility, we have used the immortalized hypothalamic GnRH neurons to determine whether these neurons can produce and respond to CBs, and have extended these studies to GnRH neurons *in vivo*.

## MATERIALS AND METHODS

### Cell culture

The immortalized hypothalamic GnRH neurons (GT1 cells) and RAW 264.7 macrophage cells were cultured and maintained in 95% $O_2$ – 5% $CO_2$ at 37°C in DMEM/F-12 medium (Invitrogen, Grand Island, NY) as described (25). For lipid studies, GT1-7 neurons were grown to 70–75% confluence in 6-well or 100 mm$^2$ plates and labeled for 18 h with either 1.5 μCi/

ml [$^3$H]ethanolamine (PerkinElmer Life and Analytical Sciences, Inc., Boston, MA) or [$^3$H] arachidonic acid (AA; PerkinElmer) in serum-free medium with 0.01% fatty acid-free BSA (Sigma-Aldrich, St. Louis, MO) and 1% N-2 supplement (Invitrogen). Following radiolabeling, neurons were washed 2X with serum-free DMEM/F-12 medium (Invitrogen) or Krebs-Ringer bicarbonate glucose buffer (KB). Neurons were subsequently stimulated for various times with ionomycin (Calbiochem, La Jolla, CA) or with norepinephrine (NE; Sigma-Aldrich) in 50 μM ascorbic acid (Sigma-Aldrich). To ascertain whether the immortalized GnRH neurons contained a CB transporter, cells were radiolabeled with [$^3$H]ethanolamine overnight and incubated in KB for 30 min in the absence or presence of the transporter inhibitor, AM 404 (Tocris Cookson Inc., Ellisville, MO). In another transporter experiment, GT1-7 neurons were incubated with 0.5 nM [$^3$H]anandamide (PerkinElmer) and 99 nM non-radioactive anandamide (ANA; Biomol, Inc., Plymouth Meeting, PA) in the absence or presence of AM 404. To determine whether the cells could degrade endocannabinoids, GT1-7 neurons were pre-labeled with [$^3$H]ethanolamine overnight and pre-incubated for 30 min in KB in the presence or absence of the fatty-acid amide hydrolase (FAAH) inhibitors: oleylethanolamide or arachidonoyl trifluoromethyl ketone (Biomol). Alternatively, cells were labeled with [$^3$H]AA overnight and stimulated with ionomycin in the absence or presence of the inhibitors. All experiments were terminated by removal of medium.

For GnRH secretion studies, approximately $6 \times 10^5$ GT1-7 cells were grown for 48 h in 24-well culture dishes as described (25). At the end of this time, neurons were washed with PBS and pre-incubated in KB for 1 h, followed by an additional incubation for 30 min, and then exposed to various agents for 30 min. To test effects of a CB receptor agonist on secretion, GT1 neurons were pre-incubated in KB in the absence or presence of different concentrations of WIN 55,212-2 (Sigma-Aldrich) or CP 55,940 (Tocris Cookson) for 30 min. Neurons were then depolarized with 56 mM [$K^+$] in the absence or presence of the agonist. To determine specificity of the agonist, cells were pre-incubated with 50 μM WIN 55,212-2 in the presence or absence of 100 μM AM 251 (Tocris Cookson), a selective CB1 receptor antagonist, and stimulated with 56 mM [$K^+$] in the presence and absence of the agonist and/or antagonist. To evaluate whether the CB receptor was coupled to Gi or Go proteins, GT1-7 neurons were incubated overnight with 0.1 μg/ml pertussus toxin (Calbiochem). The next day cells were pre-incubated with 50 μM WIN 55,212-2 and stimulated with 56 mM [$K^+$] in the presence and absence of the agonist. Finally, to ascertain whether a CB receptor agonist could influence pulsatile GnRH secretion, GT1-7 neurons were grown on Cytodex beads (Amersham Biosciences Corp., Piscataway, NJ) for 4 days (26). At the end of this time, cells/beads were loaded into 1 ml plastic syringes, perifused at 0.1 ml/min with KB continually gassed with 95% $O_2$ - 5% $CO_2$ at 37°C for 5 h, and 5-min fractions were collected over the last 4 h. Following the first 60 min of perifusion in KB, baseline secretion was assessed in the same buffer over the next 2 h. At the end of this time, one-half of the samples were perifused either with KB alone or were exposed to 50 μM WIN 55,212-2 for 30 min. Thereafter, all samples were returned to KB for 1 h. Neurons were exposed to 56 mM [$K^+$] for the last 30 min to evaluate their response to depolarization. Medium was collected and analyzed for GnRH contents by RIA (25,26).

### Animals

Adult virgin female C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were housed in a temperature- and humidity-controlled room with a 14:10 h light:dark cycle (lights on at 0700 h). Rodent chow and water were provided *ad libitum*. Animals were euthanized by decapitation for tissue samples for RNA analyses. All studies were conducted with an approved protocol from the Duke University Institutional Animal Care and Use Committee.

To evaluate effects of CBs on reproduction, estrous cycles were followed for 3–8 cycles and only females with consistent 4–6 day cycles were used. Mice were anesthetized on metestrus with 100 mg/kg ketamine - 20 mg/kg xylazine (i.p.), and implanted with a 5 mm cannula (Alzet Corp., Palo Alto, CA) into the third ventricle (AP: −0.08 mm, L: 0.0 mm, V: −4.7 mm; see 27). The cannula was attached to an Alzet minipump implanted (s.c.) along the back of the mouse. Pumps were activated with saline for 12 h before implantation and they delivered 0.5 µl/h vehicle (2% Tween-80 in artificial cerebrospinal fluid) or approximately 120 nmol/h of the CB receptor agonist, CP 55, 940 (Tocris Cookson). Animals were followed until proestrus and then methylene blue was injected into the cannula to confirm its location.

### Lipid Analyses

After stimulation, cells and medium were collected and analyzed separately or, in certain cases, pooled. Lipids were extracted from samples by sequential addition of 2.8 ml chloroform-methanol (1:2, vol/vol), 0.9 ml chloroform, and 0.9 ml distilled water (28). Samples were vortexed and centrifuged for 5 min at 400 rpm at 23°C to separate the organic and aqueous phases. The aqueous phase was aspirated and the organic phase was dried to completion under nitrogen gas. Samples were reconstituted in chloroform and loaded onto LK6D high-performance TLC plates (Whatman, Inc., Clifton, NJ). Plates were run in a solvent system of ethyl acetate – isooctane - acetic acid - water (93:47:21:100, vol/vol/vol/vol). Lipids were visualized with iodine, identified by co-migration with standards (Biomol), scraped from the plates, and quantitated by liquid scintillation counting. Alternatively, plates were sprayed with En3hance (PerkinElmer) and visualized by autoradiography with Biomax MS film (Eastman Kodak, Inc., Rochester, NY). The identity of 2-AMG and ANA were further confirmed by TLC analyses in an alternative solvent system consisting of petroleum ether - diethyl ether - acetic acid (20:80:1, vol/vol/vol) (29) or by HPLC (30). Briefly, the HPLC apparatus consisted of HP 1090 syringe pumps (Hewlett-Packard, Palo Alto, CA), a Rheodyne 7125 injector (Rheodyne, Cotati, CA), a Kratos 773 UV detector (Kratos Analytical Instruments, Ramsey, NJ), a HP 3395 integrator (Hewlett-Packard), and a Frac-200 fraction collector (Amersham). The separation was achieved on a 250 x 2.1 mm 5 µm C18 Supelcosil column (Supelco, Bellefonte, PA) with an isocratic gradient of acetonitrile-water (50:50, vol/vol) run at 1 ml/min (30). Lipids were identified according to co-elution with standards (Biomol) at 214 nm.

### Analyses of gene expression

Total RNA was isolated from GT1-1 and GT1-7 neurons, RAW 264.7 cells, as well as, various mouse tissues using TriReagent (Molecular Research Center, Inc., Cincinnati, OH). First-strand cDNA was synthesized from 5 µg DNase I-treated total RNA, using Superscript II (Invitrogen, Carlsbad, CA). After first-strand synthesis, PCR was performed. For the CB1 receptor, the 5'-GTGTGCTGTTGCTGTTCATTGTGTA-3' and 5'-GCTGTGTTATTGGCGTGCTTGT-3' primers yielded a product of ~449 bp that spanned nucleotides 1036-1483 of the murine transcript (accession #U22948). The CB2 receptor reaction was run with primers 5'-GGTCCTCTCAGCATTGATTTCTTAC-3' and 5'-TTCACATCAGCCTCTGTTTCTGTA-3' that produced a product of approximately 541 bp that included nucleotides 657-1198 of the mouse sequence (accession #009924). The FAAH reaction was run with 5'-CCTTCTTACCAAACAACATACC-3' and 5'-CCTTTTTCATGCCCTTCTTC-3' primers that produced a product of ~518 bp spanning nucleotides 1091-1609 of the mouse sequence (accession #MMU82536). The phospholipase D (PLD) primers included 5'-GCTGTAAGGATTCACATTGAC-3' and 5'-AAAAGCTCTATCATCGGTATTC-3' that yielded a product of ~201 bp that spanned nucleotides 1136–1315 of the mouse sequence (accession #NM178728). Mouse hypothalamus, pituitary, and spleen served as positive controls; negative controls included samples run without primers or with RNA from GT1-7 cells that had not undergone first-strand synthesis. The conditions for the PCR reactions consisted of an initial denaturation step at 94°C for 90

sec, followed by 40 cycles at 94°C for 40 sec, 52–58°C for 40 sec, and 72°C for 45 sec. The PCR products were separated on 2% agarose gel, verified by sequencing at the Duke University Medical Center facility, and subcloned into the pCR 2.1 plasmid using the TA cloning kit (Invitrogen).

For Northern blot analyses, total RNA from GT1-1, GT1-3, and GT1-7 cells and various mouse tissues were purified using the mRNA Purification kit (Amersham) as described (31). Ten μg mRNA was fractionated on 1.2% agarose gel and transferred to nylon membrane (Biotrans, Aurora, OH). The cDNA probes for the CB1 and CB2 receptors were radiolabeled with [α-$^{32}$P]-dCTP (PerkinElmer) by random primer and diluted to $1.2 \times 10^7$ dpm/μg DNA. Blots were hybridized, washed, and exposed to Storage Phophor Plates for visualization using a Typhoon 9200 Variable Mode Imager (Molecular Dynamics, Sunnyvale, CA).

For *in situ* hybridization histochemistry (ISHH), mouse brains were collected and frozen in powdered dry ice, then wrapped in parafilm and stored at −80°C in sealed tubes. Twelve-μm coronal cryosections were obtained from the rostral preoptic area encompassing the organum vasculosum of the lamina terminalis region through the medial preoptic area (27). Sections were affixed to gelatin-coated slides and stored at −80°C until use. The cDNA probe for the CB1 receptor was made by PCR as described above. The cDNA template for mouse GnRH (mGnRH) was a 246-bp fragment corresponding to bases 39–285 of the mGnRH cDNA (accession #AY39940) prepared using a forward primer of 5'-CGGCATTCTACTGCTGACTG-3' and a reverse primer sequence of 5'-CTTCTTCTGCCTGGCTTCCT-3'. Fragments were cloned into a TOPO-TA vector (Invitrogen) and sequenced to verify identity. Standard *in vitro* transcription was used to prepare $^{35}$S-labeled cRNA probe to the CB1 receptor and digoxigenin-labeled probes to mGnRH mRNA. Dual-label ISHH was performed as described previously (32). Sections were thawed, fixed and prehybridized, before applying a mixture of $^{35}$S-labeled cRNA probes for the CB1 receptor (1x10$^6$ cpm) and 0.5 μl of the digoxigenin-labeled mGnRH probes in 25 μl of hybridization buffer. To verify specificity, we hybridized representative sections to $^{35}$S-labeled sense strand probes in buffer with or without digoxigenin-labeled cRNA probes for mGnRH mRNA. Sections were hybridized at 52°C overnight under glass coverslips, washed and processed for immunocytochemical detection of digoxigenin-labeled probes for mGnRH mRNAs as described (32). Following detection of the dioxigenin-labeled probes, we used standard emulsion autoradiographical procedures (NTB3 emulsion; Kodak; Rochester, NY) to visualize radiolabeled probes for the CB1 receptor mRNA (7-day exposure). To analyze dual-label ISHH studies, we used BioQuant Windows (R and M Biometrics, Nashville, TN) interfaced to a Leitz Laborlux microscope through a 3CCD color video camera (Hitachi Denshi America, Ltd, Woodbury, NY). Sections from the preoptic region containing GnRH neurons (4–6 sections/animal) were examined in 5 animals and the number of GnRH neurons with and without CB1 receptor transcripts was determined.

### Statistics

The data are presented as means and SEM. Student's t-tests were used to compare two groups; other comparisons were performed by ANOVA with subsequent Dunnett-t or Bonferroni *post-hoc* tests. $P < 0.05$ was considered significant.

Pulsatile GnRH release was analyzed by several different procedures (26). First, the threshold for pulse detection was set at 3 times the coefficient of variation for assay for the concentration of that particular sample (33). Patterns of secretion were also examined using cluster analyses (34) and the DETECT program (35). Since these procedures gave similar findings, the present results are depicted from the DETECT program.

## RESULTS

### Immortalized GnRH neurons release 2-arachidonyl monoacylglycerol

As endocannabinoids are synthesized by enzymes that are activated by $Ca^{2+}$-dependent processes, many investigators use ionomycin to stimulate their production (see 20). The immortalized neurons were radiolabeled overnight with [$^3$H]AA and stimulated the next day with 5 μM ionomycin. This agent produced a time-dependent increase in 2-AMG accumulation with an enhancement observed within the first 10 min of stimulation (Fig. 1A). AA and DAG release was also augmented over the same time (Fig. 1B). To ascertain whether endocannabinoid production was responsive to other agents such as neurotransmitters, GT1-7 neurons were stimulated with 100 μM NE. The NE also released 2-AMG, DAG, and AA (Fig. 1C, D). Collectively, these data demonstrate that the immortalized GnRH neurons synthesize an endocannabinoid and that NE and other agents that increase entry of $Ca^{2+}$ into cells can stimulate release and accumulation of 2-AMG in the medium.

### Immortalized GnRH neurons release and transport anandamide

Since the neurons produce 2-AMG, we wanted to determine if they could make other endocannabinoids. Recently, a phospholipase D (PLD) species has been identified that generates the endocannabinoid, ANA (36). RT-PCR analyses and subsequent sequencing reveal that the mouse hypothalamus and GT1 cells contain transcripts for this form of CPD (Fig. 1E). No products were found when the PCR reaction was run with RNA alone or without primers.

To ascertain whether the immortalized neurons synthesized ANA, GT1-7 neurons were radiolabeled overnight with [$^3$H]ethanolamine and incubated the next day for 30 min with 100 μM AM 404, an ANA transporter inhibitor. The presence of the inhibitor alone increased ANA accumulation in medium by more than 500% (Fig. 1F). ANA was also identified by TLC and autoradiography following stimulation with 5 μM ionomycin (Fig. 1G). To determine whether ANA could be transported into immortalized GnRH neurons, [$^3$H]ANA was added to the medium and uptake was examined in the absence or presence of AM 404. In absence of the inhibitor, neurons rapidly removed the radioendocannabinoid from the medium with half-maximal uptake occurring within approximately 4 min after its addition (Fig. 1H). By comparison, AM 404 suppressed intracellular uptake of [$^3$H]ANA from the medium. Together, these findings show that the immortalized GnRH neurons synthesize ANA and can rapidly remove it from the medium.

### Immortalized GnRH neurons degrade endocannabinoids

FAAH degrades endocannabinoids in many different cells (37). To investigate whether the immortalized GnRH neurons contained FAAH transcripts, RT-PCR was performed (Fig. 2A). PCR products of appropriate size were detected not only in mouse pituitary, but also in hypothalamus -- a brain region containing GnRH neurons in vivo (24). Products of the same size were also present in the GT1 neurons. No products were found when the PCR reaction was run with RNA alone or without primers.

To study whether the FAAH was functional, GT1-7 cells were radiolabeled overnight with [$^3$H]ethanolamine and incubated the next day for 30 min with KB in the presence or absence of the enzyme inhibitors: oleylethanolamide or arachidonyl triflouromethyl ketone. Basal release of ANA was enhanced more than 180-fold by the inhibitors (Fig. 2B). To evaluate whether the enzyme could degrade 2-AMG, GT1-7 neurons were labeled with [$^3$H]AA and incubated with KB or with 5 μM ionomycin in the absence or presence of each inhibitor. Ionomycin significantly stimulated release of 2-AMG and the accumulation of the endocannabinoid was further enhanced by addition of FAAH inhibitors (Fig. 2C). Since FAAH

is an enzyme found within cells, these findings show that the GT1 neurons can remove ANA and 2-AMG from the medium and degrade these endocannabinoids.

## Immortalized GnRH neurons contain CB1 and CB2 receptor transcripts

As the immortalized GnRH neurons produced, transported, and degraded endocannabinoids, we next examined whether they contained receptors for these bioactive lipids. Northern blots were run for the CB1 receptor with lysates from mouse cerebral cortex and hypothalamus, as well as, from the three immortalized neuronal cell lines. The transcript for the CB1 receptor was ~6 kb in all samples (Fig. 3A), a size consistent with the CB1 receptor in rodents (18). By contrast, while transcripts for the CB2 receptor could be visualized in mouse spleen by Northern blot, they were not observable in mouse hypothalamus or in the three immortalized GnRH neuronal cell lines (data not shown).

Since levels of the CB1 receptor were low and concentrations of the CB2 receptor were undetectable by Northern blot, we used RT-PCR to further examine expression of these transcripts in the immortalized GnRH neurons. This more sensitive method more readily identified CB1 and CB2 receptor RT-PCR products in the GT1-1 and GT1-7 cells, as well as in mouse hypothalamus (Fig. 3B,C). Subsequent sequencing confirmed the identity of these products. Hence, the immortalized GnRH neurons contain transcripts for both CB receptors – albeit at low levels.

## Cannabinoids inhibit GnRH secretion from immortalized GnRH neurons

To determine whether the CB receptors were functional in the immortalized GnRH neurons, GT1-7 neurons were incubated in KB alone or were depolarized with 56 mM [$K^+$] in the absence or presence of a CB receptor agonist, WIN 55,212-2. The [$K^+$] stimulus augmented GnRH secretion more than 3-fold over baseline so that any inhibitory responses to CBs could be clearly visualized (Fig. 4A). WIN 55,212-2 reduced this response in a dose-dependent manner. An identical effect was observed with another CB receptor agonist, CP 55,940 (data not shown). Parenthetically, neither agonist influenced basal GnRH release (data not shown). To evaluate response specificity, the experiment was repeated in the presence of the selective CB1 receptor antagonist, AM 251. Again, 56 mM [$K^+$] stimulated GnRH secretion and this was reduced by more than 2-fold with 50 μM WIN 55,212-2 (Fig. 4B). The reduction in [$K^+$]-stimulated GnRH secretion by the agonist was blocked with 100 μM AM 251. By comparison, the CB1 receptor antagonist alone exerted no effects on [$K^+$]-stimulated GnRH release (Fig. 4B) or on basal GnRH release (data not shown). To ascertain whether the WIN 55,212-2 response was mediated by Gi/Go proteins, GT1-7 neurons were incubated in medium alone or were treated overnight with 0.1 μg/ml pertussus toxin. Although the toxin did not significantly influence GnRH secretion under basal conditions or in response to [$K^+$] stimulation (data not shown), inhibition of GnRH release by the CB receptor agonist was abrogated by the toxin (Fig. 4C). Collectively, these data demonstrate that agonist activation of CB receptors reduces [$K^+$]-stimulated GnRH secretion through a Gi/Go-mediated mechanism.

An important characteristic of immortalized GT1 neurons and GnRH neurons *in vivo* is their pulsatile release of GnRH (26,38–40). To determine whether CBs alter pulsatility, GT1-7 cells were perifused for 5 h. After 1 h of stabilization, medium was collected over the next 2 h to establish baseline. During this period, GnRH was secreted in a pulsatile manner with a frequency of 1.41±0.21 pulses/h, duration of 17.4±2.2 min, and interpulse interval of 25.3±2.8 min (Fig. 4D,E). Following exposure to 50 μM WIN 55,212-2 for 30 min, pulsatile activity ceased over the next 1 h (Fig. 4E); responses to vehicle did not affect the pattern of GnRH release (Fig. 4D). GnRH release remained very low and pulsatility was suppressed in the CB agonist-treated cultures even when they were returned to perifusion with KB (Fig. 4E). All

samples responded to 56 mM [$K^+$]-depolarization at the end of the experiment. Hence, the previously CB-treated cells can respond to depolarization; albeit not as robustly as cells treated with KB alone. These data show that a CB receptor agonist can perturb pulsatile GnRH secretion from the immortalized GnRH neurons.

### CB effects in vivo

To determine whether CBs influence GnRH function *in vivo*, a CB receptor agonist was administered to estrous cycling female mice. Animals were surgically implanted on metestrus with osmotic minipumps that delivered vehicle or low levels of the CB receptor agonist (CP 55,940) into the third ventricle of the hypothalamus, a region adjacent to the perikarya of GnRH neurons. Prior to cannulation, estrous cycles were monitored over at least 2 weeks and they were found to be approximately the same duration for both groups (vehicle controls: 5.0±0.41 days, agonist-treated mice: 4.9±0.32 days; n=7–9 females). After cannulation, vehicle controls maintained a 5-day estrous cycle, while proestrus was delayed by at least 2 days in the agonist-treated mice (Fig. 4F). These findings show that CBs can exert effects on reproduction at the level of the hypothalamus.

The effects of CBs on estrous cycle duration could be exerted on GnRH neurons themselves and/or on neurons that control GnRH function. To distinguish between these possibilities, dual-label ISHH experiments were conducted with female C57BL/6J mice. Animals were euthanized without regard to the stage of estrous cycle. An examination of a total of 117 GnRH neurons revealed that most neurons were devoid of autoradiographic grains and few grains were seen in the remaining neurons (Fig. 5A–C). Despite this fact, autoradiographic grains were prominent is areas surrounding GnRH neurons. An absence of either a GnRH or CB1 receptor signal in the sense-strand controls verified the specificity of the probes (data not shown). Hence, these findings suggest that if the CB1 receptor is expressed in GnRH neurons, it is at a very low level and most CB1 receptors are localized in neighboring cells.

## DISCUSSION

CBs have long been known to exert potent effects on reproduction (1–17). While some of these influences may be mediated at the levels of the pituitary and gonads (41–43), the primary effects have been ascribed to hypothalamic action because synthetic GnRH can rescue ovulation in CB-treated animals (1,8,13,14,17). Despite this fact, the mechanism of action of CBs on GnRH function is obscure. While ANA has been identified in hypothalamus (21), the present studies demonstrate that the immortalized hypothalamic GnRH neurons contain a complete and functional CB system. The presence of this system in the immortalized neurons, coupled with the reported *in vivo* effects of CB agonists on LH secretion and/or ovulation in female rodents and humans (1,5,6,10,13,16,17), suggests that endocannabinoids may play an important role in GnRH physiology and reproduction.

In the present report, we have found that the immortalized GnRH neurons synthesize at least two different endocannabinoids: 2-AMG and ANA. Besides endocannabinoids, $Ca^{2+}$-ionophore and NE produce corresponding increases in DAG and free AA. These latter findings are consistent with a report showing calcium-dependent hydrolysis of phosphoinositides to be associated with liberation of inositol phosphates, as well as DAG and AA in GT1-7 cells (25). In the present experiment the delayed accumulation of 2-AMG with respect to DAG suggests that this endocannabinoid is derived from phospholipid hydrolysis and is modified subsequently by DAG lipase to produce 2-AMG (44). By contrast, ANA production may proceed through the action of PLD (38,45). In this regard, it is noteworthy that the immortalized GnRH neurons possess transcripts for a PLD form that has been reported to generate ANA (36). Recently, PLD has been shown to be activated by GnRH (46). Since GnRH can inhibit its own secretion from the immortalized neurons (47) and because an ultra-short feedback

system resides on GnRH neurons *in vitro* and *in vivo* (48–50), our results suggest that endocannabinoids may play a role in this process by autocrine, paracrine or, more likely, juxtacrine interactions with cells in close apposition to GnRH neurons.

Besides synthesizing and releasing endocannabinoids, the immortalized GnRH neurons can also remove them from the medium. Under normal conditions, 2-AMG and ANA are transiently active and re-enter the cell by passive diffusion and/or active transport (51–53). While the existence of an endocannabinoid transporter is controversial (51–53), the present experiments show that GT1-7 cells can rapidly remove ANA from the medium and this action is blocked by a putative transporter inhibitor. Together these data suggest that the immortalized GnRH neurons contain a CB transporter that can remove 2-AMG and/or ANA from the medium to rapidly curtail their biological actions.

Aside from removing endocannabinoids from the medium, the immortalized GnRH neurons also degrade these lipids. Two different inhibitors of FAAH, oleylethanolamide and arachidonyl trifluoromethyl ketone, enhance 2-AMG and ANA accumulation. Interestingly, in some systems ANA is taken-up and rapidly converted to other eicosanoids, or is degraded by FAAH to AA and ethanolamine (54). The 2-AMG can be hydrolyzed to AA by either a monoacylglycerol lipase or FAAH, and re-esterified into phospholipid (55,56). Furthermore, some *in situ* studies reveal that endocannabinoids recycled into AA can be converted into eicosanoids such as thromboxanes and prostaglandins (57). Since prostaglandin E2 can stimulate GnRH secretion from median eminence tissue fragments *in vitro* (58) and from the immortalized GnRH neurons (59), their potential synthesis from endocannabinoids suggests that CBs may be intimately linked with various eicosanoid signaling pathways to modulate GnRH release.

In addition to synthesizing endocannabinoids, the immortalized GnRH neurons also contain transcripts for the CB1 and CB2 receptors and activation of these receptors inhibits GnRH secretion. In perifusion experiments, a CB receptor agonist completely disrupts pulsatile secretion of GnRH. It is noteworthy that while GnRH secretion can be stimulated with $[K^+]$ depolarization subsequent to treatment with a CB agonist, the response is less robust than in cells previously exposed to KB alone. A similar relationship is seen *in vivo* where THC is more efficient than atropine in maintaining blockade of LH responses to electrical stimulation of the preoptic area (60). Although a CB receptor agonist suppresses $[K^+]$-stimulated GnRH secretion and this response is blocked by a CB receptor antagonist in the immortalized neurons, it should be emphasized that the levels of the CB1 and CB2 receptor transcripts in the immortalized cells are low. Moreover, $[K^+]$-stimulated GnRH secretion is only partially inhibited by the highest concentration of the receptor agonist. Thus, CBs appear to exert modest effects on GnRH secretion from the immortalized neurons.

Effects of CBs were next evaluated in adult female C57BL/6J mice. A CB receptor agonist was found to prolong the estrus cycle in the mice by at least two days. These data are in agreement with other investigations where peripheral administration of CBs is reported to delay ovulation in a number of species including the human (1,5,6,10,13,16,17). There are a number of different reasons to suspect that the reproductive effects of CBs are exerted primarily at the level of the hypothalamus. First, in the present study delivery of the agonist into the third ventricle of the hypothalamus is sufficient to delay the estrous cycle in mice by several days. Second, peripherally administered GnRH restores LH secretion in CB-treated animals (8,13, 14). Third, electrical stimulation of the medial preoptic area alleviates the THC-induced blockade of the preovulatory LH surge (61). Finally, ANA is reported to depress GnRH release from the medial basal hypothalamus *in vitro* (62). Although CBs influence reproduction through hypothalamic mechanisms, it has not been clear whether these lipids exert their effects directly on GnRH neurons or on neighboring cells that control GnRH release. Importantly,

CB1 receptors are localized in the preoptic area and hypothalamus (63,64), brain areas where the GnRH neurons reside. Results from our dual-label ISHH experiments suggest that few hypothalamic GnRH neurons contain CB1 receptor transcripts. By comparison, the distribution of autoradiographic grains indicates that many cells in the vicinity of GnRH neurons possess substantial levels of CB1 receptor transcripts. Importantly, a recent report by Herbison's group (65) using single-cell PCR from transgenic mice has shown that CB2 receptors are expressed in approximately 25% of native hypothalamic GnRH neurons. Thus, these and the present findings suggest that CBs may exert their effects on reproduction not only through cells that control GnRH function, but also through GnRH neurons themselves.

GnRH neurons *in vivo* have long been known to respond to ligands that stimulate phosphoinositide hydrolysis and increased intracellular calcium and DAG production (see 25). This cascade may also lead to the synthesis of endocannabinoids, such as 2-AMG and ANA. These lipids, in turn, can bind to CB receptors on neighboring cells and GnRH neurons, thereby, exerting some feedback control on GnRH function. In this regard, it is instructive that recent electrophysiological evidence from hippocampal and cerebellar neurons indicates that CBs can send retrograde signals back to presynaptic terminals to control neurotransmitter release (66–68). If the same processes occur in GnRH neurons *in vivo*, then the generation of endocannabinoids and their degradation to various eicosanoid products could serve as a novel mechanism for regulating GnRH secretion, and ultimately, reproduction in mammals.

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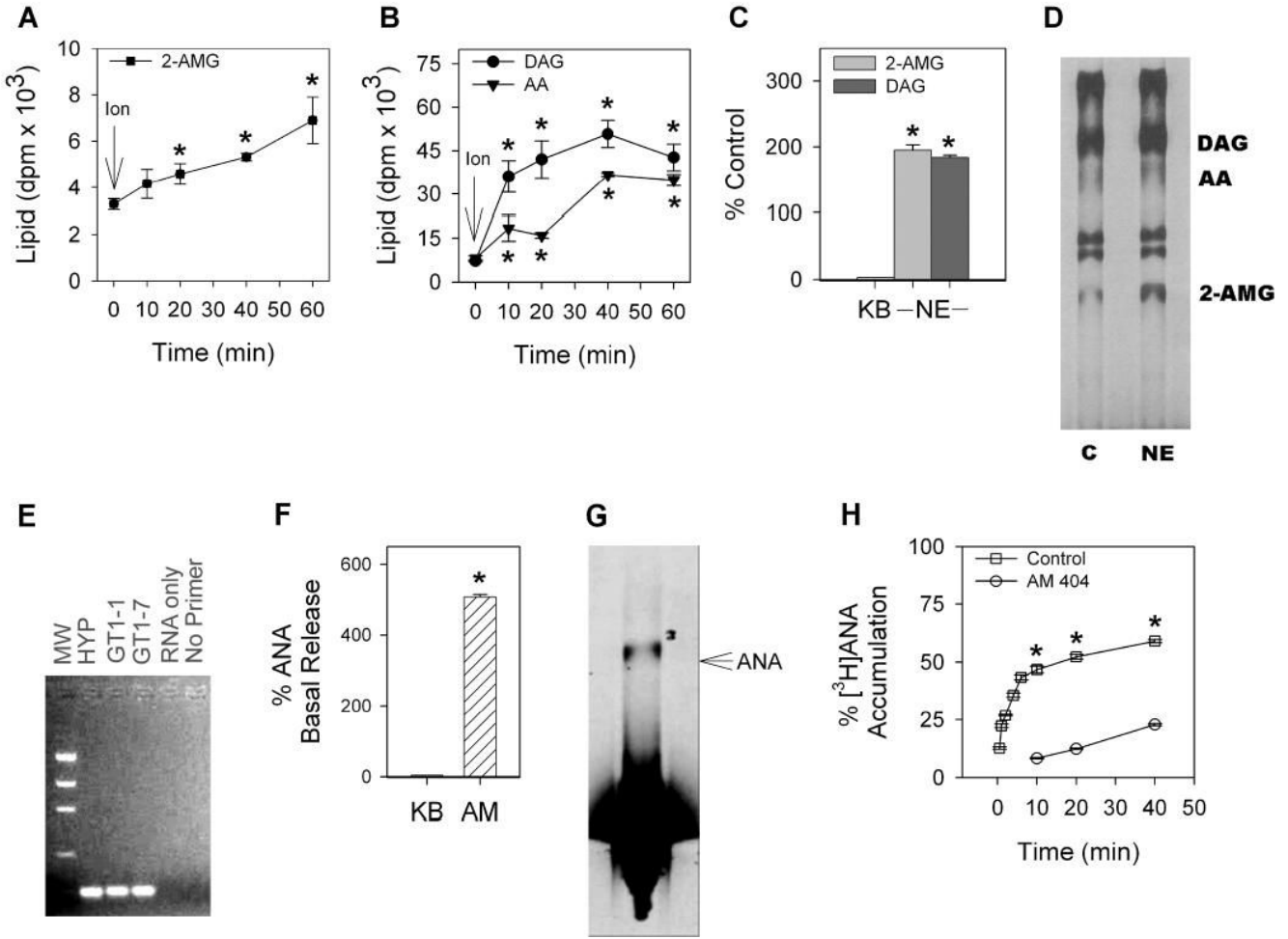
**FIG. 1.**
Immortalized hypothalamic GnRH neurons produce endocannabinoids. (A,B) Changes in release over time of 2-AMG, AA, and DAG with 5 μM ionomycin (ION). *, $P < 0.05$ from the 0 time point; n=3. (C) Percent increase over baseline of NE-stimulated release of 2-AMG and DAG over 10 min. *, $P < 0.05$ from KB; n=3–6. (D). A representative autoradiogram of DAG, AA, and 2-AMG release following incubation with the KB control (C) or stimulation with 100 μM NE for 10 min. (E) RT-PCR analyses of PLD expression in mouse hypothalamus (HYP) and GT1 cells. Lane 1: Molecular weight (MW) markers; lane 2: mouse HYP; lanes 3 and 4: samples from GT1-1 and GT1-7 neurons; lane 5: GT1-7 neuronal total RNA (no first strand synthesis); and lane 6: PCR reaction run without primers. PCR products of ~201 bp are observed. (F) Accumulation of ANA in the medium under basal conditions in the absence (KB) or presence of the CB transporter inhibitor, AM 404 (AM). #, $P < 0.05$ from KB; n=6. (F) Representative autoradiogram demonstrates ANA in medium following 5 μM ionomycin stimulation. (G) Time-course of [³H]-ANA uptake from the medium and its blockade with a transporter inhibitor. *, $P < 0.05$ from control; n= 4–14 observations.
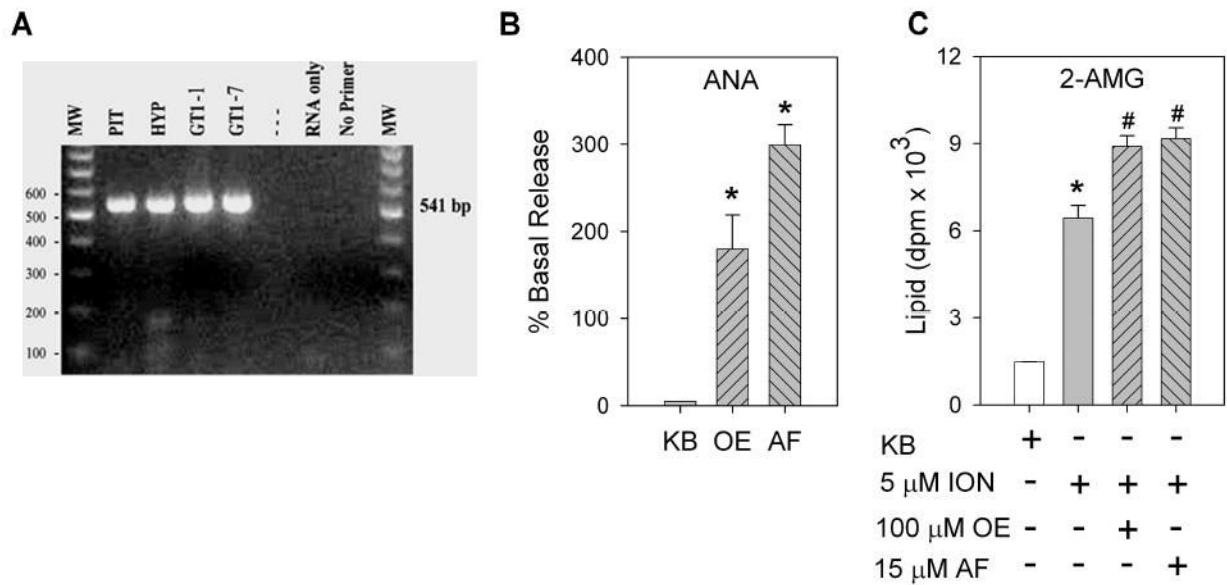
**FIG. 2.**
Immortalized GnRH neurons contain an enzyme for degrading endocannabinoids. (A) RT-PCR analyses of FAAH expression in mouse tissues and GT1 cells. Lanes 1 and 9: molecular weight (MW) markers; lanes 2 and 3: mouse pituitary (PIT) and hypothalamus (HYP); lanes 4 and 5: samples from GT1-1 and GT1-7 neurons; lane 6: blank; lane 7: GT1-7 neuronal total RNA (no first strand synthesis); and lane 8: PCR reaction run without primers. PCR products of ~540 bp are observed. (B) Basal accumulation of ANA in the absence (KB) or presence of 100 μM oleylethanolamide (OE) or 15 μM arachidonyl trifluoromethyl ketone (AF), two different FAAH inhibitors. *, $P < 0.05$ from KB; n=6. (C) Blockade of 2-AMG degradation by inhibitors of FAAH during stimulation with 5 μM ionomycin (ION). *, $P < 0.05$ KB versus ION; #, $P < 0.05$ ION alone versus OE and AF; n=3.
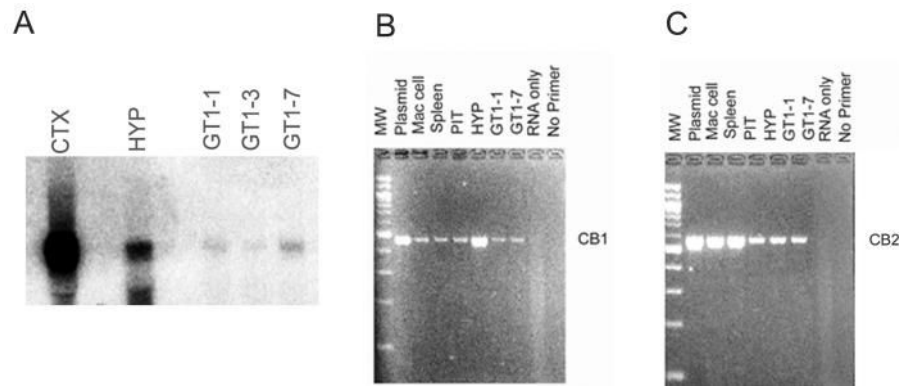
**FIG. 3.**
The immortalized hypothalamic GnRH neurons contain transcripts for CB receptors. (A) Northern blot of CB1 receptor expression in mouse tissues and GT1 neurons. Lanes 1 and 3: 10 μg poly(A)$^+$ mRNA from mouse cortex (CTX) and hypothalamus (HYP); lanes 2 and 4: blank; lanes 5–7: mRNA from the GT1-1, GT1-3, and GT1-7 neurons. The size of the mRNA is ~6 kb. (B) RT-PCR of CB1 receptor expression in various mouse tissues and cell lines. Lane 1 contains the MW markers, lane 2 was run with the mouse CB1 receptor cDNA (plasmid) as a control, lane 3 was run with RAW 264.7 macrophage (Mac) cells that are know to contain transcripts for this gene, lanes 4–6 were run with respective tissues from mouse spleen, PIT, and HYP, lanes 7 and 8 were run with GT1-1 and GT1-7 cells, lane 9 was run with total RNA (no first strand synthesis) from GT1-7 cells, and lane 10 was run without primers. The size for the CB1 receptor PCR product was ~449 bp. (C) RT-PCR of CB2 receptor expression. The same tissues and cells were used as described in the previous panel; the plasmid control was run with the mouse CB2 receptor cDNA. The size of the PCR product was ~541 bp.
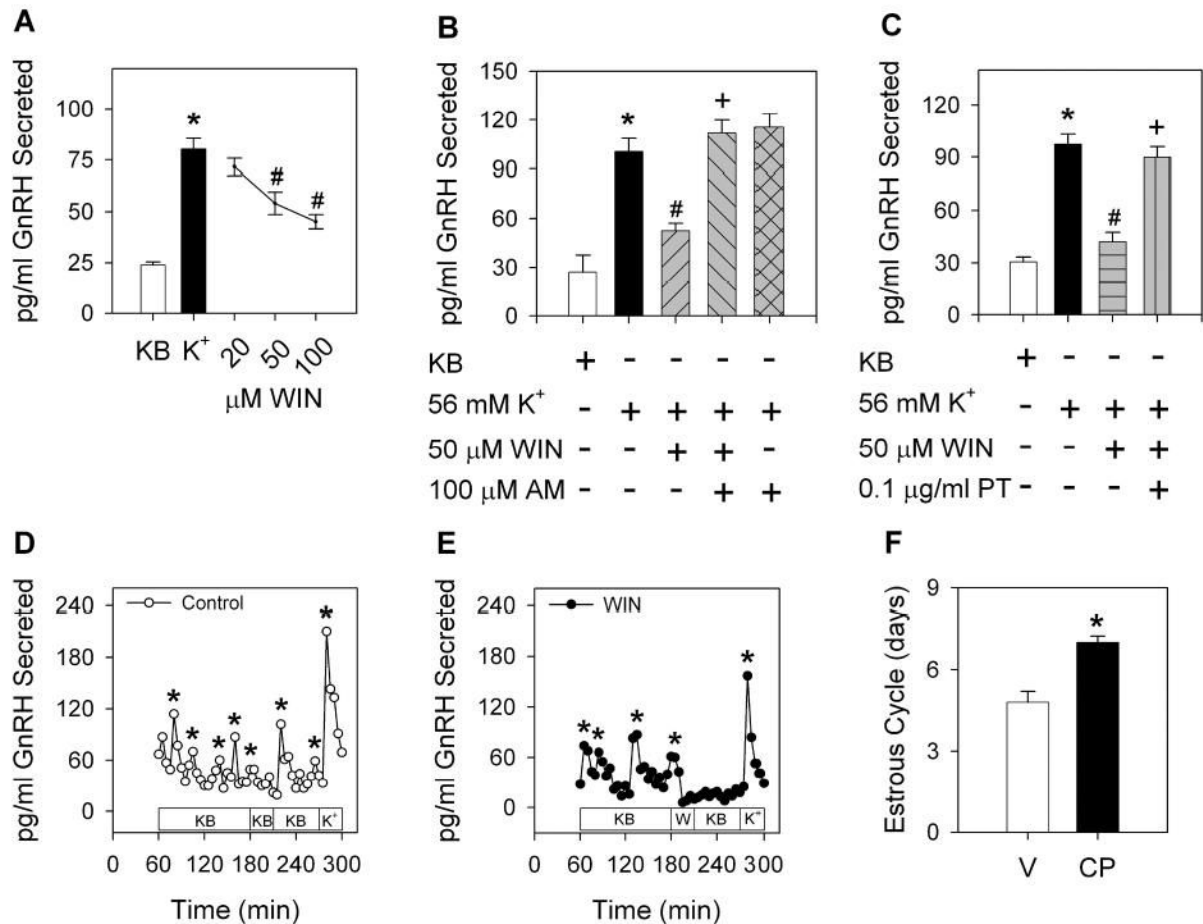
**Fig. 4.**
Immortalized hypothalamic GnRH neurons respond to CBs and these agents inhibit estrous cycles in mice. (A) The CB receptor agonist, WIN 55,212-2, reduces [K$^+$]-stimulated GnRH secretion in a dose-dependent manner. *, $P < 0.05$ [K$^+$] stimulation from KB; #, $P < 0.05$ WIN 55,212-2 from [K$^+$] stimulation; n=6. (B) Pharmacological specificity of CBs on GnRH secretion. WIN 55,212-2 decreases [K$^+$]-stimulated GnRH secretion and a CB antagonist (AM 281) blocks this effect. AM 251 alone exerts no effect on [K$^+$]-stimulated GnRH secretion. *, $P < 0.05$ [K$^+$] stimulation from KB; #, $P < 0.05$ WIN 55,212-2 from [K$^+$] stimulation; +, $P < 0.05$ AM 251 from the WIN 55,212-2 group; n=6. (C) Effects of inhibition of Gi/Go proteins on CB-mediated reduction in [K$^+$]-stimulated GnRH release. WIN 55,212-2 depresses [K$^+$]-stimulated GnRH secretion and pertussus toxin (PT) abrogates the effects of the agonist. *, $P < 0.05$ [K$^+$] stimulation from KB; #, $P < 0.05$ WIN 55,212-2 from [K$^+$] stimulation; +, $P < 0.05$ PT from WIN 55,212-2; n=6. (D,E) Representative secretory profiles from perifused immortalized GnRH neurons. After stabilization for 1 h, baseline secretion was assessed in KB over the next 2 h. At the end of this time, one-half of the immortalized GnRH neurons received KB alone while others were exposed to 50 μM WIN 55,212-2 (W) for 30 min. Thereafter, all samples were returned to KB for 1 h and were stimulated with 56 mM [K$^+$] for 30 min. *, signifies a GnRH pulse; n=10. (F) Effects of a CB receptor agonist on estrous cycles. CP 55,940 (CP) or vehicle (V) was infused into the third ventricle of regularly estrous cycling mice during metestrus. Proestrus is delayed by at least 2 days by the agonist. *, $P < 0.05$ from the vehicle control; n=7–9 mice/group.
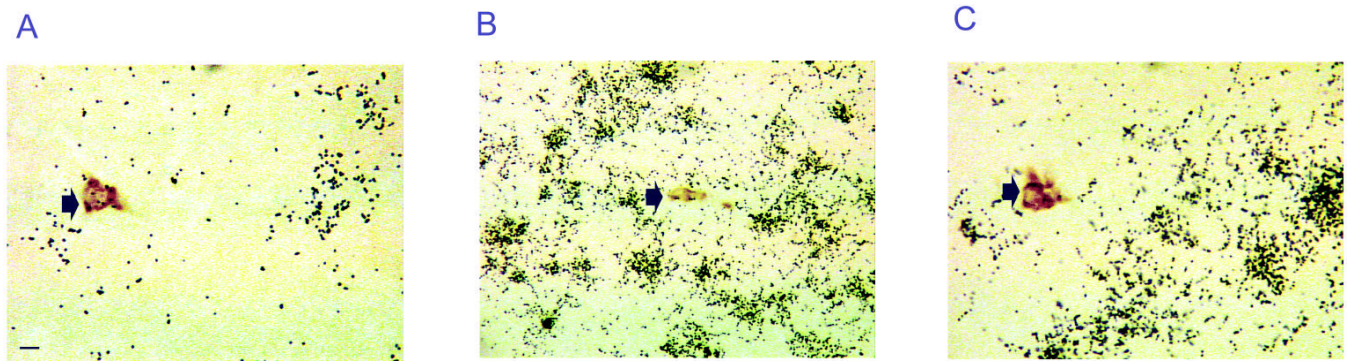
**Fig. 5.**
Analyses of CB1 receptor expression in hypothalamic GnRH neurons *in vivo.* (A–C) photomicrographs show results of dual-label *in situ* hyrbidization histochemistry of a digoxigenin-labeled cRNA for GnRH-GAP gene expression (brown stain) and a ^{35}S-labeled cRNA for expression of the CB1 receptor (black-silver grains). The arrows points to GnRH neurons. All photomicrographs are at 40X objective. Scale bar in panel A: 5µm.