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Flufenamic acid modulates multiple currents in gonadotropin-releasing hormone neurons

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Abstract

Reproduction in mammals is dependent upon the appropriate neurosecretion of gonadotropin-releasing hormone (GnRH), yet the endogenous generation of activity underlying GnRH secretion remains poorly understood. We have demonstrated that the depolarizing afterpotential (DAP), which modulates bursting activity, is reduced in isolated GnRH neurons from aged animals. Calcium-activated non-specific cation (CAN) channels contribute to the DAP in other vertebrate neurosecretory cells. We used the CAN channel blocker flufenamic acid (FFA) to examine the contribution of CAN channels to the DAP in GnRH neurons during aging. Recordings were performed on isolated fluorescent GnRH neurons from young, middle-aged and aged female mice. Flufenamic acid inhibited spontaneous activity, but significantly increased the DAP in neurons from young and middle-aged animals. Apamin did not significantly potentiate the DAP, but did reduce the effects of FFA, suggesting that the increased DAP is partially due to blockade of apamin-sensitive SK channels. Flufenamic acid increased the current underlying the DAP (I_{ADP}) and decreased the preceding fast outward current (I_{OUT}) at all ages. These current responses were not affected by apamin, but TEA evoked similar changes. Thus, a potassium current, likely mediated through BK channels, contributes to the fast AHP and appears to offset the DAP; this current is sensitive to FFA, but insensitive to age. The effect of FFA on the DAP, but not I_{ADP} , is diminished in aged animals, possibly reflecting an age-related modulation of the apamin-sensitive SK channel. Future studies will examine the expression of SK channels during the aging process in GnRH neurons.

Keywords

FFA; GnRH; calcium-activated non-specific cation channels; potassium channels; aging

1. Introduction

Gonadotropin-releasing hormone (GnRH) neurons are the pivotal neural control center in a tightly regulated reproductive axis. The release of GnRH is pulsatile, resulting in the release of gonadotropic hormones (follicle stimulating hormone [FSH] and luteinizing hormone [LH]) from the anterior pituitary. This pulsatile release of GnRH is necessary for the maintenance of normal reproductive function in both males and females, and is likely dependent upon neuronal activity (Wilson et al., 1984, Hiruma et al., 1994). However, many questions concerning the endogenous generation and exogenous regulation of GnRH pulsatile activity remain unanswered.

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Burst firing is a hallmark of neurosecretory cells (Dutton and Dyball, 1979, Bicknell et al., 1982); GnRH neurons fire in bursts of several action potentials (Suter et al., 2000, Kuehl-Kovarik et al., 2002, Nunemaker et al., 2002, Nunemaker et al., 2003). Depolarizing afterpotentials (DAPs) contribute to burst firing in GnRH neurons (Kuehl-Kovarik et al., 2005, Chu and Moenter, 2006), as they do in magnocellular neurosecretory cells (Armstrong et al., 1994, Li and Hatton, 1997, Ghamari-Langroudi and Bourque, 2002). A number of currents modulate the DAP in GnRH neurons. A TTX-sensitive, but uncharacterized, sodium current contributes to DAP amplitude (Chu and Moenter, 2006). Apamin-sensitive (Lee et al., 2010), and possibly iberiotoxin sensitive (Chu et al., 2009) currents appear to attenuate the DAP. Calcium-activated non-specific cation (CAN) channels contribute to the DAP in magnocellular neurons (Ghamari-Langroudi and Bourque, 2002) and hippocampal CA1 neurons (Partridge and Valenzuela, 2000). The CAN channel is calcium dependent and shows little voltage dependence. Channels discriminate poorly between sodium and potassium, yet underlie slow depolarizing events in a number of vertebrate and invertebrate neurons (Partridge and Swandulla, 1988).

In recent years, it has become clear that a number of changes are occurring at the level of the hypothalamus (Wise et al., 2002) during reproductive aging. Both human females and female rodents experience a preovulatory rise in FSH (Wise, 1999, Rubin, 2000, Wise et al., 2002) before the transition to reproductive senescence. This is followed by a decreased LH pulse frequency (Hall et al., 2000, Rubin, 2000, Wise et al., 2002), variable cycle length (Wise et al., 2002), and an attenuated LH response (Wise et al., 2002) to estradiol. The number of GnRH pulses decreases in middle-aged female rats during a steroid-induced LH surge (Rubin and Bridges, 1989, Rubin, 2000), but it is not clear why this decrease occurs. We have recently shown that DAP amplitude is reduced in isolated GnRH neurons from aged animals (Wang et al., 2008). In addition, spontaneous activity and patterning is significantly reduced with increasing age (Wang et al., 2008). An attenuated DAP amplitude could lead to the decreased frequency and loss of spike patterning seen in GnRH neurons from old animals, potentially resulting in dysregulated GnRH release. In an effort to characterize age-sensitive currents that contribute to the DAP, we examined the effect of flufenamic acid (FFA), a CAN channel blocker, on the DAP and underlying current (I_{ADP}) in GnRH neurons.

2. Results

Effect of FFA on cell parameters

We have shown previously (Wang et al., 2008) that age does not affect membrane capacitance or input resistance in GnRH neurons, although there is a trend towards decreased capacitance with increasing age. Input resistance is affected by hormone status (Wang et al., 2008). The application of flufenamic acid (FFA; 100 μ M) did not affect input resistance (control = 2.09 ± 0.17 G Ω , FFA = 1.97 ± 0.14 G Ω , $p = 0.36$, $n = 12$).

Effect of FFA on activity

Flufenamic acid (100 μ M) was bath applied to isolated GnRH neurons during cell attached recordings. All cells fired spontaneously before application of FFA. Variability in rate and pattern of firing was noted (Wang et al., 2008). However, most recordings exhibited the quick bursting pattern that is characteristic of GnRH neurons (Figure 1). Analysis of firing activity before application of FFA demonstrated that hormone treatment had no effect on firing parameters (Wang et al., 2008), but age significantly decreased firing rate (Wang et al., 2008). The application of FFA completely inhibited firing in 69% of GnRH neurons (Figure 1), and slowed firing (by greater than 10%) in another 18%. These effects could be washed out (Figure 1). Inhibition by FFA predominated in every age and treatment group

(percent responding: young OVX = 90%; young OVX+E = 91%; MA OVX = 83%; MA OVX+E = 100%; old OVX = 100%; old OVX+E = 56%). When apamin (100 nM), a blocker of small-conductance potassium channels (SK), was added to the control and drug solution, FFA still inhibited firing in a majority of neurons (5 of 7; 71%) and slowed firing by 29% in one neuron (14% of overall neurons), results very similar to FFA alone. Apamin alone did not inhibit firing ($n = 7$), although it did slow firing by 15% in one neuron.

Effect of FFA on DAPs and I_{ADPs}

Depolarizing current pulses were delivered at 3.5 ms, 200–220 pA, to evoke a single action potential, or 200 ms, 15–30 pA, to evoke a burst of action potentials. In addition, single action potentials were simulated with an 80-mV, 2-ms square voltage pulse. The effects of flufenamic acid were assessed on responses to these protocols, including depolarizing afterpotentials (DAPs) following a single spike or burst of spikes, spike number during a 200-ms depolarization, and inward and outward currents in response to the voltage pulse. Flufenamic acid did not affect spike number during the 200-ms depolarization (control = 3.44 ± 0.23 , FFA = 3.43 ± 0.18 , $p = 0.63$, $n = 38$), although spike number was affected by age and hormone treatment (Wang et al., 2008). The addition of FFA had no effect on holding current (control = -25.7 ± 3.0 pA; FFA = -20.7 ± 2.9 pA; $p = 0.20$, $n = 38$).

Flufenamic acid significantly increased DAP amplitude following a single action potential (Figure 2A, B; RM ANOVA; $F(2,91) = 16.5$; $p < 0.001$ when compared to control; $n = 38$) or a burst of action potentials ($p < 0.001$ RM ANOVA on Ranks, $n = 45$). The effect of FFA on DAP amplitude in GnRH neurons was examined at three ages (young, middle aged and old), and with (E; OVX+E) and without (CO; OVX) estradiol treatment in the animal. Flufenamic acid significantly increased the DAP only in young animals and middle aged OVX animals (Figure 2C; RM ANOVA; $p < 0.03$, $n = 6-8$). The amplitude of the DAP in FFA was significantly larger in young animals than in other age groups ($p = 0.001$, ANOVA, $F(2,33) = 8.4$, $n = 12-14$). As the effect of FFA was not different between OVX and OVX+E animals ($p = 0.38$), data within an age group was combined. In combined groups, the effect of FFA was, again, significant in young animals ($p < 0.001$ by RM ANOVA; $n = 14$) and middle aged animals ($p = 0.002$; $n = 12$) but not old animals ($p = 0.13$; $n = 12$). When the percent change in FFA was compared between groups, the percent increase was significantly smaller in old animals compared to middle aged animals (Figure 2D; ANOVA on Ranks, $p = 0.04$, $n = 12-16$ /group).

Flufenamic acid inhibits a wide variety of calcium-activated currents. Thus, FFA could have its effects through the inhibition of apamin-sensitive small-conductance potassium channels (SK). Blocking the outward calcium-activated potassium current with apamin (100 nM; Figure 3A, C; Apa/control) did not significantly increase the amplitude of the DAP, and was significantly different from FFA/control ($p = 0.02$; Table 1). However, applying apamin to the bath prior to the application of FFA reduced the potentiating effects of FFA from 167% to 124% (FFA+Apa/Apa; Figure 3C; Table 1), an increase that was no longer significant (Apa vs. FFA+Apa, $p = 0.40$, $n = 6$).

The ability of FFA to affect latency to peak DAP amplitude was examined following a 200-ms depolarizing pulse or single action potential. Neither flufenamic acid nor apamin significantly affected latency (Table 1).

The apamin-sensitive current that affects DAP amplitude is proposed to do so by modulating the afterhyperpolarization potential (AHP; Chu et al., 2009, Lee et al., 2010). Flufenamic acid significantly decreased the AHP of the single action potential ($p < 0.001$, RM ANOVA on Ranks; $n = 38$). There was no significant difference when percent change was compared for the different age and treatment groups. Although apamin alone did not significantly

reduce the AHP ($p = 0.64$, $n = 7$), the percent reduction in AHP with apamin application (Apa/control) was significant ($p < 0.05$) when compared to FFA+Apa/Apa while FFA, with outward currents blocked by apamin, had no effect (Figure 3B,D; Table 1).

To examine the effects of FFA on the currents underlying the DAP, FFA was applied during a single simulated action potential in voltage clamp. In agreement with current-clamp data, FFA significantly increased the amplitude of the current underlying the DAP (I_{ADP} , Figure 4A₁, B; RM ANOVA, $F(2,51) = 25.4$, $p < 0.001$; $n = 40-56$). In addition, FFA significantly decreased the fast outward current preceding the I_{ADP} (I_{OUT} , Figure 4A₂, C), that is presumed to contribute to the action potential afterhyperpolarization (RM ANOVA, $F(2,51) = 22.4$, $p < 0.001$; $n = 40-56$). Flufenamic acid appeared to increase the amplitude of the I_{ADP} at every age and treatment, although significance was only reached in estradiol-treated and young animals (Figure 4D; RM ANOVA, $p < 0.02$ in young, OVX+E animals, $n = 10-11$). Despite the apparent effect of estradiol when amplitudes were compared, there was no effect of age or treatment on the percent increase in FFA (Figure 4F; Age: $p = 0.85$, $F(2,50) = 0.17$; Treatment: $p = 0.27$, $F(1,50) = 1.2$; Two-way ANOVA, no interactions, $n = 7-11$ /group). The effect of FFA on the I_{OUT} was apparent at essentially every age and treatment (Figure 4E), and was not influenced by age (Figure 4G; ANOVA on Ranks, $p = 0.58$, $n = 17-19$) or treatment (Rank Sum Test, $p = 0.15$, $n = 24-30$).

Effect of potassium and calcium channel blockers

Interestingly, blocking the outward calcium-dependent potassium current with apamin prior to the addition of FFA (FFA+Apa/Apa) did not reduce the potentiation of the I_{ADP} , or the reduction of I_{OUT} seen with the addition of FFA alone (Figure 5; Table 1). Apamin alone (Apa/control) had no effect on the I_{ADP} (Figure 5A, Table 1), although apamin + iberiotoxin increase the I_{ADP} in the slice preparation (Chu et al., 2009). The percent increase in I_{ADP} amplitude in FFA+Apa/Apa was significantly larger than Apa/control ($p < 0.001$; Table 1). Similarly, apamin alone did not decrease I_{OUT} (Figure 5B, Table 1), and the reduction of I_{OUT} by FFA+Apa/Apa was significant when compared to the effects of apamin alone ($p < 0.001$; Table 1). Because the amplitude of I_{OUT} appeared to influence the amplitude of I_{ADP} , the relationship between I_{OUT} and I_{ADP} was further defined. Tetraethylammonium chloride (TEA; 5 mM), was administered as a broad-spectrum potassium channel blocker; cadmium chloride (0.5 mM) was applied to inhibit calcium currents. The application of 5 mM TEA in isolated neurons resulted in a significantly decreased I_{OUT} (Figure 5B; $p < 0.001$, RM ANOVA on Ranks, $n = 19$) and a corresponding increase in I_{ADP} (Figure 5B; $p = 0.01$, RM ANOVA on Ranks, $n = 19$). Conversely, cadmium significantly increased I_{OUT} ($p = 0.01$, RM ANOVA, $n = 16$), and had no effect on I_{ADP} (Figure 5B; $p = 0.30$, RM ANOVA on Ranks, $n = 16$).

Effect of TTX on I_{ADP}

Previous data obtained from slice recordings indicate that I_{ADP} , the current underlying the DAP, is sensitive to TTX (Chu and Moenter, 2006). Although I_{ADP} does not change with age (Wang et al., 2008), the amplitude of the DAP does decrease with increasing age in isolated GnRH neurons (Wang et al., 2008). Thus the sensitivity of I_{ADP} to TTX (0.5 μ M) was compared in neurons from young and aged animals to determine if age influenced TTX-sensitive sodium currents underlying the I_{ADP} . Tetrodotoxin virtually abolished the I_{ADP} in neurons isolated from both old and young animals (Figure 6; young, $p < 0.001$, $n = 6$; old, $p = 0.014$, $n = 5$, RM ANOVA).

Effect of high TEA on the action of FFA

Both FFA and TEA increase I_{ADP} amplitude. To determine how potassium currents contribute to the effects of FFA, the fast outward current was abolished with a high TEA,

reduced sodium solution (110 mM TEA; (Raman and Bean, 2001). Because the I_{OUT} was abolished by this solution, the measurement of I_{ADP} was taken at the time of the average I_{ADP} peak in normal ACSF, determined to be 19.6 ± 1.22 ms after the onset of the depolarizing stimulus. Based on this number, an average of the response was taken at 18–22 ms post-stimulus (Figure 7A). The amplitude of I_{ADP} was decreased in the high TEA solution (Fig 7A; $p < 0.001$, $n = 17-21$), likely due to decreased sodium in the high TEA solution. When FFA was applied in the presence of high TEA, the amplitude of the I_{ADP} did not increase, rather it decreased significantly relative to the control solution (Fig. 7B,C; RM ANOVA, $F(2,58) = 11.69$, $p < 0.001$ high TEA control compared to FFA or wash; $n = 17-21$). However, this effect did not wash out.

Effect of FFA on action potential characteristics

In examining the effects of FFA on the DAP, it was noted that FFA appeared to affect action potential width. Therefore, characteristics of the action potential were examined for modulation by FFA or apamin. The application of FFA had no effect on action potential amplitude (control = 117.6 ± 2.3 mV, FFA = 115.6 ± 2.3 mV; $p = 0.56$; t-test, $n = 37$). However, a significant increase was seen in action potential width (at half amplitude) with the addition of FFA (Figure 8A,B, also see 3B; $p < 0.001$, ANOVA on Ranks, $n = 16-36$). Flufenamic acid significantly increased action potential width at half amplitude in both young and old animals ($p < 0.04$, $n = 6-8$), and there was no difference with age or treatment (Figure 8D; Age: $p = 0.22$, $F(2,30) = 1.6$; Treatment: $p = 0.40$; $F(1,30) = 0.72$; Two-way ANOVA, no interactions, $n = 6-8$ / group).

The application of apamin alone (Apa/con) resulted in no increase in action potential width (Figure 8C); the percent change in apamin (Apa/con) was significantly different than the percent change in FFA (FFA/con; $p = 0.002$, $n = 6-9$). However, blocking SK channels prior to the application of FFA (F+A/Apa) did reduce the effects of FFA (Figure 8C; Table 1) such that the increase in width with FFA application was no longer significant (FFA vs. control, $p = 0.003$, $n = 9$; FFA+Apa vs. Apa, $p = 0.16$, $n = 7$).

Effect of FFA on whole-cell currents

FFA was applied during 12-ms voltage steps to examine effects on inward and outward whole-cell currents. FFA reduced both inward (Figure 9A,B) and outward (Figure 9A,C) currents. The reduction in whole-cell currents by FFA was evident in every age and treatment group (Figure 9D,E). Flufenamic acid significantly reduced mean inward current density in every treatment group ($p \leq 0.004$, RM ANOVA, $n = 8-14$ per group), and there were no effects of age or treatment (Age: $p = 0.31$, $F(2,61) = 1.2$; Treatment: $p = 0.59$; $F(1,61) = 0.29$; Two-way ANOVA, no interactions, $n = 8-14$ / group), although there was an effect of estradiol on control currents (Wang et al., 2008; Figure 9D; $p < 0.002$, Two-way ANOVA, $F(1,61) = 10.0$, $n = 31-36$, no interactions). The application of FFA also slightly, but significantly reduced mean outward current density in nearly all groups (Figure 9E; $p < 0.05$, RM ANOVA, $n = 8-14$). Again, there was no significant difference between treatments (Age: $p = 0.98$, $F(2,61) = 0.02$; Treatment: $p = 0.18$; $F(1,61) = 1.86$; Two-way ANOVA, no interactions, $n = 31-36$ / group).

Inward current density in FFA was 62% of control, and was reduced to 39% of control by the addition of apamin to the bath (Table 1). As expected, apamin alone had no effect on inward current density, and the percent change in apamin alone was significantly different from FFA or FFA+Apa ($p < 0.001$; Table 1). Apamin alone had no effect on outward current density, although outward current density was reduced by FFA alone (Table 1). The reduction of outward current by FFA was significant when compared to apamin alone ($p = 0.002$; Table 1).

Effect of FFA on sodium currents

Because FFA decreased whole-cell inward currents, the effect of FFA on a specific inward current, the sodium current underlying the action potential, was examined. Flufenamic acid had a similar effect on the sodium current underlying both the simulated and the real action potential (Figure 10). The TTX-sensitive current (Wang et al., 2010) was decreased significantly under both protocols (evoked: Figure 10B; $p < 0.001$, $n = 9-16$; simulated: Figure 10A; $\text{Con} = -1026 \pm 47$ pA/pF, $\text{FFA} = -717 \pm 29$ pA/pF, $p < 0.001$, $n = 66$). Apamin had no significant effect on the inward sodium current ($\text{Con} = -333 \pm 26$ pA/pF, $\text{apamin} = -293 \pm 24$ pA/pF, $p = 0.267$, $n = 20$).

Overlaying current traces with and without FFA application suggested that the time-course of inactivation was different between the two treatments (Figure 10A,C). Inactivation was best fit with a single exponential function. The inactivation time constant (τ ; Tau) was significantly prolonged at peak currents (Figure 10C; $p < 0.05$; $n = 8-11$) with FFA application.

3. Discussion

These results demonstrate that the application of 100 μM flufenamic acid, recognized as a blocker of calcium-activated non-specific cation channels (CANs), increases the depolarizing afterpotential, and the underlying current (I_{ADP}) in adult GnRH neurons. In addition, FFA increases action potential width, yet inhibits spontaneous activity and decreases whole-cell and sodium currents. Thus, it appears that FFA can have multiple, often incongruous effects in GnRH neurons. Flufenamic acid (300 μM) has been shown to have paradoxical effects in mollusk neuroendocrine cells as well, evoking both a calcium-dependent I_{in} and a TEA-sensitive I_{out} (Gardam et al., 2008). In GnRH neurons, FFA reduces both an inward and outward voltage-gated current, indicating that some effects of FFA appear to be mediated through voltage-gated channels, rather than the calcium-activated channels that were targeted in these studies.

Apamin (100 nM) in the bath reduced the potentiation of the DAP seen with FFA alone, but did not eliminate it. Conversely, the effect of FFA on the AHP was abolished by apamin. Apamin-sensitive, small-conductance, calcium-activated potassium channels, or SK channels (Kato et al., 2006, Spergel, 2007), are known to affect the medium and/or slow AHP in a variety of neurons (Vogalis et al., 2003). Rodent GnRH neurons have a relatively slow, apamin-sensitive I_{AHP} (Kato et al., 2006, Lee et al., 2010) that offsets the DAP (Chu et al., 2009, Lee et al., 2010), as seen in the current study. However, our results demonstrate that SK channels do not wholly account for the potentiating effects of FFA on DAP amplitude, suggesting that another slow calcium-activated current is also antagonized by FFA. A potential candidate current is a very slow, UCL2077-sensitive, calcium-activated afterhyperpolarizing current (Lee et al., 2010). This current has not yet been shown to offset the DAP in GnRH neurons (Lee et al., 2010).

Interestingly, apamin had no effect on I_{ADP} or I_{OUT} , the fast current preceding I_{ADP} . It has recently been shown that a 600-ms, 100-mV depolarizing voltage step is required to fully activate the apamin-sensitive I_{AHP} in GnRH neurons, and that depolarizing for 100 ms would only have evoked 30% of the peak AHP current (Liu et al., 2008). Thus, our voltage protocol (2 ms), although physiological, may have been too short to detect apamin-sensitive currents in our model. High TEA (110 mM) abolished both I_{OUT} and the effect of FFA on I_{ADP} , and both FFA and TEA (5 mM) reduced I_{OUT} , while increasing the amplitude of I_{ADP} . Our results suggest that another FFA-sensitive potassium channel contributes to I_{OUT} , offsetting I_{ADP} . One candidate would be the large conductance calcium-activated channel, BK, although FFA has also been shown to inhibit the voltage-activated transient outward

potassium current (I_A) in cultured cerebellar granule cells (Zhao et al., 2007). The decay time constant for I_{OUT} was 1.98 ± 0.155 ms ($n = 58$ from all ages), suggesting that I_{OUT} underlies the fast AHP. The fast AHP results from the activation of voltage-gated and/or fast calcium-activated potassium channels, including BK, that contribute to action potential repolarization (Faber and Sah, 2003); most are sensitive to TEA (Faber and Sah, 2003). The slow AHP offsets the DAP (Chu et al., 2009, Lee et al., 2010); it is interesting that this fast I_{AHP} influences the amplitude of I_{ADP} as well. It appears that multiple FFA-sensitive potassium channels contribute to various components of the AHP, and, further, impact the amplitude of the subsequent depolarizing afterpotential.

Similar to the effect of FFA on DAP amplitude, FFA increases action potential width through both apamin-sensitive and apamin-insensitive currents. The small, but significant effect of FFA on the sodium current inactivation time constant could increase action potential width. However, blockade of calcium-activated BK channels with charybdotoxin has been shown to increase action potential duration in rat GnRH neurons (Hiraizumi et al., 2008), suggesting that the effect of FFA on action potential duration is through the blockade of both BK and SK channels. Although not modulated by aging, future studies with specific blockers of BK channels are warranted.

Results indicate that the potentiation of the DAP by FFA is age specific. A significant portion of the DAP appears to be mediated through a TTX-sensitive sodium channel (Chu and Moenter, 2006), as the underlying I_{ADP} is eliminated by TTX application (Chu and Moenter, 2006). However, our results demonstrate that this channel is not modulated during the aging process. The apamin-insensitive current underlying the fast AHP (I_{OUT}) is FFA sensitive, but is also not modulated by aging. Our results suggest that the apamin- and FFA-sensitive SK current, not detected by our voltage protocols, is affected by age in GnRH neurons. Future experiments will closely examine the effect of age on SK currents and expression in GnRH neurons.

In summary, FFA increases DAP and I_{ADP} amplitude, likely through the blockade of potassium channels that contribute to both the fast and slow AHP. An increase in action potential duration, also seen with FFA application, may be modulated through these same channels. The apamin-insensitive current is not modulated by age, yet the DAP is, suggesting that SK channels may be age-sensitive in GnRH neurons. In addition, FFA inhibits spontaneous activity and decreases sodium currents. Future studies will address the ionic bases behind these findings.

4. Experimental procedures

Animals

Adult virgin female GnRH-eGFP transgenic mice (Suter et al., 2000, DeFazio and Moenter, 2002, Kuehl-Kovarik et al., 2002, Wang et al., 2008) were used for all experiments. Animals were maintained in a colony at the University of Missouri, under a 12 hour light:dark cycle. Food (LabDiet 5008; PMI Nutrition International, Richmond, IN) and water were available at all times. All animal experimentation was conducted in accord with accepted standards of humane animal care, and the University of Missouri Animal Care and Use Committee approved all procedures. Mice were taken at three months of age to represent young reproductive females, 10 months of age (middle aged, MA), representing the peripostreproductive phase, and 15–18 months of age (old), postreproductive animals (Wang et al., 2008). Young adults were used in all studies involving apamin. At least three mice were used in each treatment group. All mice were bilaterally ovariectomized (OVX) under isoflurane anesthesia five to seven days prior to sacrifice. Banamine (0.025 mg/10 g body weight) was administered pre-operatively as an analgesic. At the time of surgery, a

SILASTIC brand capsule (0.052 in. ID, 0.125 in. OD; Dow Corning, Midland, MI) was placed subcutaneously. The capsule either contained 0.625 μg β -estradiol (E_2 ; OVX+E; Sigma, St. Louis, MO) in 20 μl of tocopherol-stripped corn oil (MP Biomedicals; Solon, OH) or corn oil only (OVX). This amount of E_2 results in significantly increased serum estradiol and significantly higher uterine weights in estradiol-treated animals (Wang et al., 2008). Mice were sacrificed 5–7 days after steroid replacement to allow sufficient time for the endocrine adjustment to hormone manipulation while avoiding long-term steroid withdrawal (Nunemaker et al., 2002).

Acute dissociation of neurons

Cells were acutely isolated as described previously (Kuehl-Kovarik et al., 2002, Wang et al., 2008). This protocol has been designed specifically to allow the physiological analysis of neurons from aged brains (Kuehl-Kovarik et al., 2003) as the preparation of neurons suitable for electrophysiological studies becomes very difficult with increased age. Briefly, brains were quickly removed and sliced at 400 μm in low-calcium artificial cerebrospinal fluid (ACSF; (Wang et al., 2008)). Isolated regions were enzymatically treated with proteinase K (Sigma; 0.2 mg/ml) followed by trypsin (Sigma Type XI; 1 mg/ml) at 30°C for 24–31 minutes, depending upon the lot of trypsin and the age of the animal. Because older brains contain more connective tissue, slices from the older animals (10–18 month) were trypsinized for 2–3 minutes longer than the young animals, to produce healthy isolated neurons at all ages (Kuehl-Kovarik et al., 2003, Wang et al., 2008). Slices were triturated with flame-polished Pasteur pipettes and dispersed cells were incubated overnight in Neurobasal A/B-27 (Life Technologies, Inc., Carlsbad, CA) before electrophysiological recordings. No estradiol was added to the dish after dissociation.

Electrophysiology

Electrophysiology was performed 16–24 hours post-dissociation, as described previously (Kuehl-Kovarik et al., 2002, Wang et al., 2008). Cells were perfused with normal-calcium ACSF (2.5 mM CaCl_2) at 22°C. Recordings were obtained with thin-walled borosilicate glass micropipettes (World Precision Instruments, Sarasota, FL; 1.5–3 M Ω) filled with a potassium gluconate intracellular solution (in mM: 120 potassium gluconate, 1 CaCl_2 , 1 MgCl_2 , 10 HEPES, 1 NaCl, 5 EGTA, 2 ATP, 0.2 GTP, pH 7.2–7.4). All recordings were performed using a MultiClamp 700B amplifier (Molecular Devices, Sunnyvale, CA), digitized with a Digidata 1322A (Molecular Devices), and stored on a Dell computer using pClamp 9.2 software.

Cell-attached recordings were performed to examine the effect of FFA on spontaneous firing in cells isolated from young and aged animals. Recordings were initiated with a seal >300 M Ω , although most seals were >1 G Ω by the end of the recording. Recordings were initiated ~30 seconds after the establishment of the seal. Signals were acquired at 5 kHz and low-pass filtered at 1 kHz. The duration of the recordings lasted for 15–40 minutes.

Whole-cell configuration was initiated only upon the establishment of a G Ω seal. Only stable recordings with a series resistance of less than 10 M Ω and a stable membrane capacitance were included. Series resistance was compensated 50–90%. A liquid junction potential of –10 mV was not corrected. Whole-cell current-clamp recordings were performed to examine action potential responses and resulting depolarizing afterpotentials (DAPs; Kuehl-Kovarik et al., 2002, Kuehl-Kovarik et al., 2005, Chu and Moenter, 2006). The majority of cells were firing (resting membrane potential < –50 to –55 mV) when whole-cell, current-clamp mode was achieved. Cells were hyperpolarized to –55 mV to prevent spontaneous activity; cells requiring > 20 pA of current to hyperpolarize below threshold were discarded. Depolarizing current pulses were delivered at 3.5 ms, 200–220

pA, to evoke a single action potential, or 200 ms, 15–30 pA, to evoke a string of action potentials. Data was digitized at 20 kHz and low-pass filtered at 4 kHz.

Whole-cell voltage-clamp recordings were made to examine the currents underlying the DAP (I_{ADP} ; Chu and Moenter, 2006). Whole-cell currents and currents evoked by an action potential were also examined. Cells were clamped at -60 mV. Single action potentials were simulated with an 80-mV, 2-ms square voltage pulse (Mitterdorfer and Bean, 2002, Chu and Moenter, 2006). The stimulus was repeated 35 times at 1-s intervals, and responses were averaged. Whole-cell inward and outward currents were evoked with a 12-ms, 10-mV per step depolarizing voltage protocol. To measure the ionic current underlying the action potential, a pre-recorded spike train was used as the voltage command. Tetrodotoxin (TTX; 0.5 μ M; Sigma) was used to isolate the sodium current. All amplitude measurements were taken in reference to the prepulse baseline. Latency and duration measurements were taken from the time of pulse initiation.

Flufenamic acid (FFA; Sigma; 100 μ M; Ghamari-Langroudi and Bourque, 2002) was prepared as a stock solution in dimethyl sulfoxide (DMSO; Sigma) and added to the drug solution. DMSO was added to the control solution at a final concentration of 0.2% . Apamin (Apa; Sigma; 100 nM; Ghamari-Langroudi and Bourque, 2002, Kato et al., 2006) was added as a test drug, or to both the drug and control solution. All compounds were applied for at least two minutes before recordings were initiated, and typically applied for a total of 5–10 minutes. Compounds were washed out for 3–10 minutes before data collection.

Data Analysis

Data was generated with Clampfit 9.2 and SigmaStat (Systat) software. For whole-cell and sodium currents, current densities for each cell were calculated as current (pA) divided by capacitance (pF). Differences between control and drug applications were compared with repeated measures ANOVAs (RM ANOVAs) or RM ANOVA on Ranks. Comparisons between responses to drug treatments, or different ages and hormone treatments, were made with 1- and 2-way ANOVAs and unpaired t-tests (as appropriate) for normally distributed data, and by Kruskal-Wallis ANOVA on ranks and Mann-Whitney rank sum tests for nonparametric data. Post-hoc analyses (Holm-Sidak, Dunn's) were performed when appropriate. All values are expressed as mean $\pm$ SEM.

Abbreviations

AHP	afterhyperpolarization potential
CAN	calcium-activated, non-specific cationic channel
DAP	depolarizing afterpotential
E	estradiol
eGFP	enhanced green fluorescent protein
FFA	flufenamic acid
GnRH	gonadotropin releasing hormone
I_{ADP}	inward current underlying the DAP
I_{OUT}	fast outward current preceding the I_{ADP}
OVX	ovariectomized
RM ANOVA	repeated measures analysis of variance

SK	small-conductance, calcium-activated potassium channel
TEA	tetraethylammonium chloride: potassium channel blocker
TTX	tetrodotoxin; sodium channel blocker

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Figure 1. Flufenamic acid inhibits firing activity in GnRH neurons. Representative cell attached recording from a GnRH-eGFP neuron isolated from a 10-month old, OVX+E mouse. The application of 100 μ M FFA is shown in gray.

Figure 2.

Flufenamic acid affects DAP amplitude. **(A)** Representative whole-cell current-clamp traces (averaged from 3–5 records, young OVX+E animal), recorded from a GnRH neuron in a) control solution, b) flufenamic acid (FFA, 100 μM), and c) after wash, are overlaid. Action potentials (cropped) were evoked by a 3.5-ms, 200-pA current pulse. **(B)** The depolarizing afterpotential (DAP) amplitude following a single action potential is significantly increased (*: $p < 0.001$ when compared to control, $n = 38$, all animals combined) by the application of 100 μM FFA. **(C)** The amplitude of the DAP is significantly increased by FFA only in young and middle-aged (MA) animals (a: $p < 0.03$ as compared to control in that age/treatment group, $n = 6–8$). The amplitude of the DAP in 100 μM FFA is significantly larger in young animals than other age groups (b: $p = 0.001$ when compared to FFA in middle-aged, old group, $n = 12–14$). **(D)** When comparing the amplitude percent change (FFA/control), the percent increase is significantly decreased in old animals (*: $p = 0.04$ compared to middle-aged animals, OVX and OVX+E combined, $n = 12–16$). CO = cells from OVX (control) animal; E = cells from OVX+E animal.

Figure 3.

The effect of FFA on the DAP and AHP are partially blocked by apamin. **(A)** Representative whole-cell current-clamp traces (averaged from 3–5 records), recorded from a GnRH neuron isolated from a 3 month OVX animal. Traces in control solution and apamin (100 nM) are overlaid. **(B)** Representative whole-cell current-clamp traces on a different scale. The afterhyperpolarization potential (AHP) is reduced by apamin, but FFA in the presence of apamin does not markedly reduce the AHP. Note the widening of the action potential by the application of FFA. **(C)** Adding apamin to the control solution (FFA+Ap/Apa) reduces the potentiation of the DAP by FFA such that the increase in FFA is no longer significant ($p = 0.40$; $n = 6$). However, apamin alone (Ap/con) does not change the amplitude of the DAP ($p = 0.67$; $n = 7$), nor the amplitude of the control solution containing apamin ($p = 0.87$, $n = 6–10$). FFA alone significantly increases the DAP in young animals (*: FFA vs control; $p < 0.001$, $n = 10$). **(D)** Apamin significantly decreases the amplitude of the AHP (*: $p < 0.05$ when compared to F+A/Apa, $n = 6–9$) following the action potential. FFA preceded by apamin (F+A/Apa) has no effect on the AHP.

Figure 4.

Flufenamic acid affects I_{ADP} and I_{OUT} amplitude. (**A**) A representative current response (cropped) to a 2-ms, 80-mV voltage pulse (trace = average of 35 records), recorded from a GnRH neuron in the presence of control or flufenamic acid (FFA, 100 μ M). A slow inward current (I_{ADP} , **A₁**) is preceded by a fast outward current (I_{OUT} , **A₂**, same trace on a different scale). (**B**) The I_{ADP} amplitude is significantly increased (*: $p < 0.001$ when compared to control or wash, $n = 40$ –56, all animals combined) by the application of FFA. (**C**) The amplitude of I_{OUT} is significantly reduced by the application of FFA (*: $p < 0.001$ when compared to control or wash, $n = 40$ –56, all animals combined). (**D**) A significant increase in I_{ADP} with FFA application is seen in all age groups (a: $p < 0.02$, $n = 10$ –11; MA = middle-

aged). (**E**) A significant decrease in I_{OUT} is seen in almost all groups (a: $p < 0.03$, $n = 10-11$). No effect of age on I_{ADP} (**F**) or I_{OUT} (**G**) is noted when comparing the amplitude percent change (FFA/control). However, the I_{OUT} displays a large variability, both between and within an age group. CO = cells from OVX (control) animal; E = cells from OVX+E animal.

Figure 5.

TEA increases I_{ADP} while decreasing I_{OUT} . (**A**) As seen for FFA, TEA (5 mM) significantly increases I_{ADP} amplitude (a: TEA vs control; $p = 0.01$, $n = 19$). Neither apamin nor cadmium affect I_{ADP} . (**B**) Similar to FFA, TEA (5 mM) significantly decreases I_{OUT} (a: TEA vs control; $p < 0.001$, $n = 19$). Apamin alone does not reduce the amplitude of I_{OUT} , and cadmium significantly increases I_{OUT} amplitude (b: cadmium vs control; $p = 0.01$; $n = 16$).

Figure 6.

Tetrodotoxin significantly reduces I_{ADP} amplitude in isolated GnRH neurons from young and old animals. (A) A representative current response to a 2-ms, 80-mV voltage pulse (trace = average of 35 records), recorded from a GnRH neuron in the presence or absence of tetrodotoxin (TTX, 0.5 μ M). The I_{ADP} is essentially abolished in the presence of TTX. (B) The I_{ADP} is significantly reduced in both young and old animals by the addition of TTX (a: $p < 0.02$ compared to control at both ages; $n = 5-6$ OVX animals per group).

Figure 7.

FFA does not increase I_{ADP} amplitude in high TEA solution. **(A)** Representative current responses (cropped) to a 2-ms, 80-mV voltage pulse (trace = average of 35 records) recorded from a GnRH neuron in normal solution (ACSF) or high TEA (110 mM). The arrow demonstrates where amplitude was measured for all experiments performed in 110 mM TEA, as peak amplitude could not be determined in this solution. The high TEA completely abolishes I_{OUT} , and results in a significantly smaller I_{ADP} ($p < 0.001$; $n = 17-21$). **(B)** Representative current responses (cropped) demonstrating that FFA does not increase I_{ADP} amplitude in high TEA. **(C)** In a high TEA solution, the I_{ADP} amplitude is significantly

reduced by FFA (*: $p < 0.001$ in control compared to other treatments, $n = 17-21$), but the effect of FFA does not wash out.

Figure 8.

Flufenamic acid increases action potential width in GnRH neurons. **(A)** Representative whole-cell current-clamp traces (averaged from 3–5 records), recorded from a GnRH neuron in control solution, flufenamic acid (FFA, 100 μ M), and after wash, are overlaid. Action potentials were evoked by a 3.5-ms, 200-pA current pulse. **(B)** The width of a single evoked action potential is significantly increased (*: $p < 0.001$ when compared to control, $n = 36$) by the application of FFA. **(C)** The application of apamin alone does not increase action potential width (control vs. apa, $p = 0.9$, $n = 7$; *: $p = 0.002$ when compared to FFA/con, $n = 6–9$). Adding apamin to the bath does insignificantly reduce the potentiation seen with FFA, and the increase in width with the addition of FFA is no longer significant ($p = 0.16$, $n = 7$). **(D)** The action potential width is increased by FFA in both young and old animals (a: $p < 0.04$ as compared to control in that age/treatment group, $n = 6–8$; MA = middle aged). There are no age or treatment differences. CO = cells from OVX (control) animal; E = cells from OVX +E animal.

Figure 9.

Flufenamic acid decreases whole-cell currents in GnRH neurons. (A) Representative traces of the current response to a series of 12-ms voltage steps (shown below control response) in control solution, flufenamic acid (FFA, 100 μ M), and after wash. Note the reduced inward and outward currents during drug application. (B) Current density-voltage curve of mean inward currents in young animals, demonstrating the effect of FFA and *in vivo* estradiol on inward currents. Current density-voltage curves were similar for middle-aged and old animals. (C) A current density-voltage curve of mean outward currents in young animals demonstrates an effect of FFA on outward currents. The figure legend in (C) applies to (B) as well. The legend indicates the animal treatment (OVX or OVX+E) and the *in vitro*

treatment of the neuron (control or FFA). Similar effects were apparent in curves generated for middle-aged and old animals. **(D)** An analysis of mean inward current density at 0 mV reveals that FFA significantly reduces the mean inward current density in all age and treatment groups (a: $p \leq 0.004$ as compared to the control in each group, $n = 8-14$; MA = middle-aged). In addition, neurons isolated from OVX+E animals demonstrate significantly smaller currents in control solution (b: $p = 0.002$ when compared to OVX age-matched controls, $n = 31-36$). The effect of FFA is not age-dependent ($p = 0.98$, $n = 20-26$). **(E)** An analysis of mean outward currents at 50 mV reveals a significant decrease at all ages (a: $p < 0.05$ when compared to control in that age/treatment group, $n = 8-14$). There is no effect of age or treatment on outward current densities. CO = cells from OVX (control) animal; E = cells from OVX+E animal.

Figure 10.

Flufenamic acid has multiple effects on sodium currents underlying the action potential. **(A)** Representative current responses to a simulated action potential voltage command. Traces demonstrate that FFA reduces, but prolongs the current response. **(B)** Sodium current density, in response to an evoked action potential command, is significantly reduced by FFA application in all age and treatment groups (*, $p < 0.001$, $n = 9-16$; MA = middle-aged). **(C)** The inactivation time constant τ is significantly prolonged after FFA application in virtually all age and treatment groups (*, $p < 0.05$, $n = 8-11$). CO = cells from OVX (control) animal; E = cells from OVX+E animal.

Table 1

Effect of flufenamic acid (FFA, 100 μ M), flufenamic acid preceded by apamin (FFA+Apa/Apa,) and apamin alone (Apa/con) on depolarizing afterpotentials and currents in isolated GnRH neurons.

Property (% of control)	FFA/ control	FFA+Apa/ Apa	Apa/ control
Depolarizing Afterpotential			
Amplitude (mV)	167.0 $\pm$ 16.3%	124.2 $\pm$ 12.6%	105.1 $\pm$ 10.3% ^a
	n=10	n=6	n=7
Latency (ms)	100.3 $\pm$ 5.0%	98.1 $\pm$ 10.0%	105.4 $\pm$ 12.0%
	n=14	n=6	n=7
I _{ADP} Amplitude (pA)	134.2 $\pm$ 6.0%	161.0 $\pm$ 10.3%	96.5 $\pm$ 4.2% ^b
	n=19	n=8	n=18
I _{OUT} Amplitude (pA)	40.0 $\pm$ 4.9%	31.0 $\pm$ 7.5%	154.8 $\pm$ 28.3% ^b
	n=19	n=8	n=18
Action Potential			
AHP Amplitude (mV)	77.5 $\pm$ 17.5%	98.4 $\pm$ 14.4%	72.6 $\pm$ 10.2% ^c
	n=9	n=6	n=7
Width (1/2 amp, ms)	150.6 $\pm$ 6.1%	127.3 $\pm$ 12.9%	106.3 $\pm$ 5.8% ^d
	n=9	n=6	n=7
Current Density			
Inward (pA/pF)	62.2 $\pm$ 3.4%	38.8 $\pm$ 4.2% ^e	98.0 $\pm$ 2.2% ^e
	n=26	n=8	n=16
Outward (pA/pF)	82.9 $\pm$ 3.1%	77.9 $\pm$ 4.5%	98.2 $\pm$ 3.2% ^f
	n=26	n=8	n=16

^{a)}Significantly different from FFA/control; p = 0.02, ANOVA.

^{b)}Significantly different from FFA+Apa/Apa; p<0.001, ANOVA.

^{c)}Significantly different from FFA+Apa/Apa; p<0.05, T-test

^{d)}Significantly different from FFA/control; p = 0.002, ANOVA.

^{e)}Significantly different from other treatments; p<0.001, ANOVA.

^{f)}Significantly different from other treatments; p= 0.002, ANOVA.