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An unidentified yet notable modification on I_{Na} and $I_{K(DR)}$ caused by ramelteon

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Abstract

Despite advancement in anti-seizure medications, 30% of patients continue to experience recurrent seizures. Previous data indicated the antiepileptic properties of melatonin and its agonists in several animal models. However, the underlying mechanisms of melatonin and its agonists on cellular excitability remain poorly understood. In this study, we demonstrated the electrophysiological changes of two main kinds of ion channels that are responsible for hyperexcitability of neurons after introduction of melatonin agonists- ramelteon (RAM). In Neuro-2a cells, the amplitude of voltage-gated Na^+ (I_{Na}) and delayed-rectifier K^+ currents ($I_{K(DR)}$) could be suppressed under RAM. The IC_{50} values of 8.7 and 2.9 μM, respectively. RAM also diminished the magnitude of window Na^+ current ($I_{Na(W)}$) elicited by short ascending ramp voltage, with unchanged the overall steady-state current–voltage relationship. The decaying time course of I_{Na} during a train of depolarizing pulses arose upon the exposure to RAM. The conditioning train protocol which blocked I_{Na} fitted the recovery time course into two exponential processes and increased the fast and slow time constant of recovery the presence of RAM. In pituitary tumor (GH_3) cells, I_{Na} amplitude was also effectively suppressed by the RAM. In addition, GH3-cells exposure to RAM decreased the firing frequency of spontaneous action potentials observed under current-clamp conditions. As a result, the RAM-mediated effect on INa was closely associated with its ability to decrease spontaneous action potentials. Collectively, we found the direct attenuation of I_{Na} and $I_{K(DR)}$ caused by RAM besides the agonistic action on melatonin receptors, which could partially explain its anti-seizure activity.

KEYWORDS
current kinetics, delayed rectifier K^+ current, melatonin, ramelteon, voltage-gated Na^+ current, window Na^+ current

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Po-Ming Wu and Yi-Fang Tu are the co-first authors of this study.

1 | INTRODUCTION

Epilepsy is defined as recurring, unprovoked seizures occurring at least 24 h apart.[1] It is one of the common neurological disorders affecting both adult and children.[2] If left untreated, it often correlates with cognitive dysfunction, particularly in patients with refractory epilepsy.[3,4] Despite advancements in currently available anti-seizure medications (ASMs), up to 30% of individuals experience treatment failure with the first-line ASMs and continue to have seizures.[2,5] These patients often necessitate the use of multiple ASMs, yet such combinations often lead to various adverse effects, including cognition decline and psychiatric symptoms. There is a demand for ASMs that are both effective and exhibit fewer adverse effects for epilepsy patients.

ASMs reduce the probability of seizure occurrence by modifying action potentials of neurons and reducing synchronization in localized neuronal networks.[6] At least part of these effects were through the modulation of voltage-gated ion channels, including sodium, calcium, and potassium channel, which were associated with neuronal excitability.[6–8] The Na$^+$ currents (I_{Na}), representing the activity of voltage-gated Na$^+$ (Na$_V$) channels, are responsible for generating and propagating action potentials in excitable membranes.[9,10] These channels rapidly transition from the resting to the open state upon brief depolarization and then briefly shift to the inactivated state. Conversely, the voltage-gated K$^+$ (K$_V$) channels play another essential role in determining cell membrane excitability. The delayed rectifier K$^+$ current ($I_{K (DR)}$) is associated with action potential firing in various types of excitable cells,[11–18] primarily originating from the K$_V$3.1-K$_V$3.2 subtypes of K$^+$ channels. The biophysical properties of K$_V$3.1 or K$_V$3.2 currents are characterized by a positively shifted voltage dependency and by rapid deactivation rate.[18,19] Furthermore, increased K$_V$-channel activity has been observed to attenuate the cumulative inhibition of I_{Na} elicited during high-frequency spike firing, thereby facilitating high-frequency synaptic transmission.[11–13,16,20] The combined effects if these two major currents on neuronal cells might lead to seizures.[21]

In addition to their role in maintaining circadian rhythms governing the normal sleep–wake cycle,[22–24] a growing body of evidence suggests melatonin and its agonists possess anti-seizure effects.[25,26] Although the anti-seizure properties of melatonin agonists have been demonstrated in experimental studies,[27] they have not been confirmed in most clinical trials as adjunctive treatments for epilepsy.[28] Therefore, there is an urgent need to explore the underlying mechanisms of melatonin and its agonists on cellular excitability. Among all synthetic melatonin analogue, ramelteon (RAM), a highly selective melatonin

agonist for human melatonin-1 (MT1) and melatonin-2 (MT2) receptor,[29,30] has piqued our interests as the first FDA-approved melatonin analogue, displaying the ability to reduce seizure frequency in previous animal studies.[27]

Based on the aforementioned considerations, experiments were designed to determine whether and how melatonin agonists and other relevant compounds could modify various types of ionic currents in Neuro-2a and pituitary GH$_3$ cells, thereby implicating the potential anti-seizure effect of these compounds on neuronal cells beyond melatonin receptors. These two cells were included in our experiments because both are electrically excitable cells, and the biophysical properties of various ionic currents in these cells have been well understood at our laboratory.[14,15,19,31,32] Previous studies have reported that melatonin can modify Na$^+$ current in different cell types,[33–36] including dorsal root ganglion neurons.[35] However, the effects of RAM on ionic currents remained poorly understood.

2 | MATERIALS AND METHODS

2.1 | The chemical compounds and solution used in this study

For this study, RAM was acquired from MedChemExpress (Bio-Genesis, Taipei, Taiwan), melatonin (5-methoxy-N-acetyltryptamine,N-acetyl-5-methoxytryptamine), tefluthrin (Tef), and tetraethylammonium chloride (TEA) were from Sigma-Aldrich (Merck, Taipei, Taiwan), while ProTx-II (a selective inhibitor of NaV1.7 sodium channels) was from Tocris (Genechain, Kaohsiung, Taiwan).

The ionic compositions of the external solution (normal Tyrode's solution) used in the present experiment were the same as our previous work and the detail was provided in Supplementary data 1.[37,38]

2.2 | Cell preparations for Electrophysiological recordings

We purchased Neuro-2a (N2a) and GH$_3$ cells from the Bioresources Collection and Research Center ([BCRC-60026, BCRC-60015], Hsinchu, Taiwan).In brief, we maintained Neuro-2a cells in DMEM supplemented with 2 mM L-glutamine, 10% (v/v) heat-inactivated fetal bovine serum, 1.5 g/L sodium bicarbonate, 1.0 mM sodium pyruvate, and 0.1 mM non-essential amino acids.[39] GH$_3$ cells were cultured in Ham's F-12 medium supplemented with 15% (v/v) horse serum, 2.5% (v/v), and 2 mM L-glutamine.[31,32] After 60%–80% confluence of cultured cells, we performed the measurements.

2.3 | Patch-clamp recordings: electrophysiological measurements

We harvested Neuro-2a or GH$_3$ cells with a 1% trypsin–EDTA solution before each measurement, and an aliquot of cell suspension was placed into a homemade chamber securely positioned on the working stage of a DM-IL inverted microscope (Leica; Major Instruments, Kaohsiung, Taiwan). The details of measurements, data recordings and data analyses were succinctly presented in Supplementary data 2.

2.4 | Statistical analyses of curve fitting approximations and experimental measurements

To conduct global least-squares analyses for linear or nonlinear curve fitting, we primarily employed the "Solver" add-in program embedded in Excel® 2021 for curve-fitting procedures. However, for validation purposes, we occasionally utilized the 65-bit OriginPro® 2021 software (OriginLab®; Scientific Formosa, Kaohsiung, Taiwan) for double-checking.[40,41] The values of experimental measurements were presented as the mean ± standard error of mean (SEM).The experiments were repeated with independent sampling in several cells. Student's t-test and analysis of variance (ANOVA) followed by post-hoc Fisher's least significant difference test were used to detect statistical significance of different groups. $p < 0.05$ was considered significant between groups (indicated with [+], **, or, [+] in the figures or table).

3 | RESULTS

3.1 | The modifications of voltage-gated Na$^+$ current (I_{Na}) by ramelteon (RAM) in Neuro-2a cells

Firstly, we wanted to explore if the I_{Na} magnitude identified in these cells can be modified by adding RAM. As demonstrated in Figure 1A, when RAM was administered to the external solution, the amplitude of peak I_{Na} was markedly attenuated in N2A cells. Figure 1A revealed that the I_{Na} amplitude was respectively attenuated to 2956 ± 212 pA ($n = 8$, $p < 0.05$) or 1984 ± 183 pA ($n = 8$, $p < 0.05$) from a control value of 3341 ± 234 pA ($n = 8$) at a concentration of 3 or 10 μM RAM and returned to 3226 ± 223 pA ($n = 8$) after removal of RAM.

Figure 1B shows that the addition of RAM can dose-dependently decrease the peak amplitude of I_{Na} elicited by rapid depolarizing command. The optimal IC$_{50}$ value required for RAM-induced block of peak I_{Na} observed in
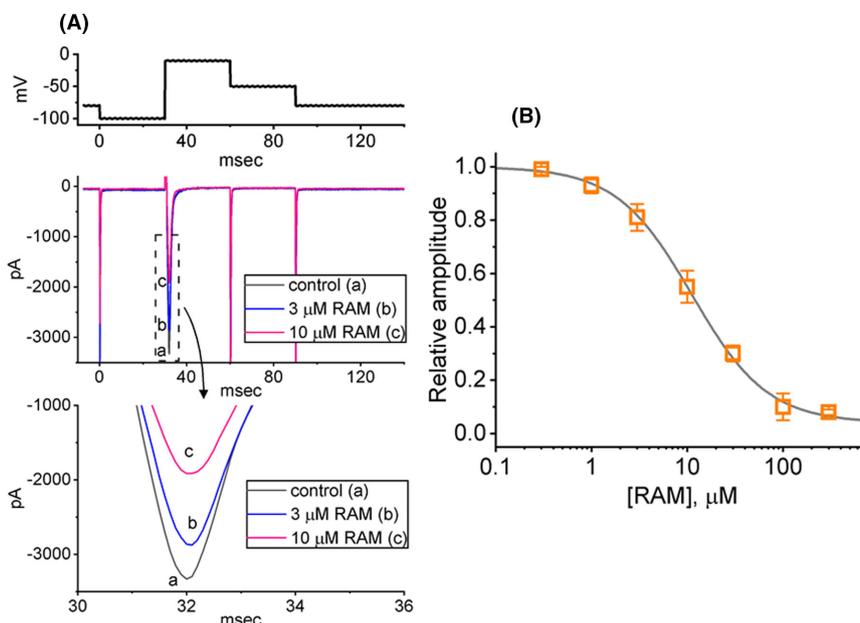

FIGURE 1 Effect of ramelteon (RAM) on voltage-gated Na$^+$ current (I_{Na}) identified in Neuro-2a cells. In this stage of experiments, we kept cells to be bathed in Ca^{2+}-free, Tyrode's solution which contained 10 mM tetraethylammonium chloride (TEA) and 0.5 mM CdCl$_2$, and for the recordings, we filled up the electrode with a Cs$^+$-containing solution. The composition of bathing or filling solution was described in Section 2. (A) Representative current traces (i.e., I_{Na}) acquired in the absence (a, black color) and during cell exposure to 3 μM RAM (b, blue color) or 10 μM RAM (c, pink color) in a patch-clamped cell. The lower part in (A) indicates the expanded record from dashed box in the middle part, while the uppermost part is the voltage-clamp protocol given. (B) Concentration-dependent inhibition of RAM on the peak amplitude of I_{Na} residing in Neuro-2a cells (mean ± SEM; $n = 8$ for each point). The gray smooth line overlaid on the data points (orange squares) is the best fit to a modified Hill equation as described in Section 2, having an IC$_{50}$ value of 11.2 μM.

Neuro-2a cells was $11.2\,\mu M$. The present experiments revealed that the RAM exposure can exert a depressant action on I_{Na} intrinsically expressed in Neuro-2a cells.

3.2 | Effect of RAM on the steady-state current versus voltage (I–V) relationship of peak I_{Na}

Because of the depressant effect of RAM, we questioned if RAM could perturb steady-state I–V relationship of I_{Na} in Neuro-2a cells. Figure 2 depicts the average I–V relationship of I_{Na} acquired with or without RAM. In the control, $G = 74.1 \pm 2.1$ nS, $V_h = -19.1 \pm 0.3$ mV, and $k = 7.6 \pm 0.7$ ($n = 7$), and in the presence of $10\,\mu M$ RAM, $G = 41.8 \pm 1.9$ nS, $V_h = -18.8 \pm 0.3$ mV and $k = 7.5 \pm 0.7$ ($n = 7$). These observations clearly revealed that the addition of RAM can result in a substantial reduction of I_{Na} conductance (G value) and that the overall I–V relationship of I_{Na} remains unaltered during exposure to this compound.
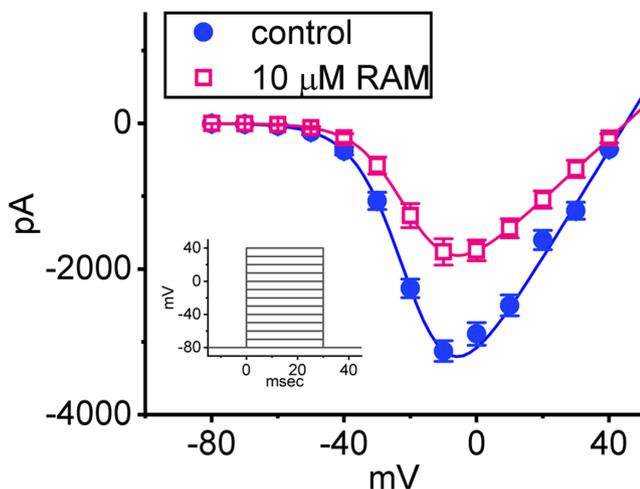

FIGURE 2 Effect of RAM on the current versus voltage (I–V) relationship of peak I_{Na} in Neuro-2a cells. These measurements were undertaken when the tested cell was held at the level of -80 mV and a series of voltage pulses ranging between -80 and $+40$ mV with a duration of 30 ms was applied to evoke I_{Na}. We filled up the recording electrode with a Cs^+-containing solution, and cells were placed in Ca^{2+}-free, Tyrode's solution containing 10 mM TEA and 0.5 mM $CdCl_2$. The average I–V relationships of peak I_{Na} in the absence (filled blue circles) and presence (open pink squares) were constructed and they are hence presented here. Current amplitude was measured at the start of short depolarizing command from -80 mV to various membrane potentials. Inset shows the voltage-clamp protocol imposed over the tested cell. Each point represents the mean $\pm$ SEM ($n = 7$). Continuous blue or pink line in which data points were overwritten shows least-squares fit of the modified Boltzmann equation (detailed in Section 2).

3.3 | RAM supressed window component of I_{Na} ($I_{Na(W)}$) in Neuro-2a cells

We next explored if the presence of RAM is able to inhibit $I_{Na\,(W)}$ magnitude elicited by short ascending ramp pulse (V_{ramp}), which appears under non-equilibrium conditions as previously reported.[42] Figure 3 demonstrated that as cells were continuously exposed to RAM, the total area (Δarea) of $I_{Na(W)}$ measured at a range of -40 and $+40$ mV was substantially reduced. RAM decreased the $I_{Na(W)}$'s Δarea to 17.7 ± 2.4 mV·nA ($n = 8$, $p < 0.05$) or 15.2 ± 2.1 mV·nA ($n = 8$, $p < 0.05$) from a control value of 21.2 ± 3.0 mV·nA ($n = 8$) at a concentration of 3 or $10\,\mu M$, respectively. On the other hand, melatonin ($30\,\mu M$) alone had minimal effect on $I_{Na(W)}$ magnitude induced by the ascending V_{ramp}.

3.4 | RAM-induced enhancement in cumulative inhibition of I_{Na} in Neuro-2a cells

The inactivation of I_{Na} has been previously reported to accumulate during repetitive short pulses in different cell types.[7,43–47] In the control period, the I_{Na} amplitude elicited by a 1-s repetitive depolarization from -80 to -10 mV was progressively decayed in a single exponential process with a time constant of 119 ± 11 ms ($n = 8$) (Figure 4A,B). The protocol represents the occurrence of an accumulative inactivation of I_{Na} in response to a train of depolarizing pulses. It is of particular interest that the decaying time course of I_{Na} during the same train of depolarizing pulses was shortened with a time constant of 93 ± 9 ms ($n = 8$, $p < 0.05$) or 79 ± 7 ms ($n = 8$, $p < 0.05$) when exposure to 3 or $10\,\mu M$ RAM, respectively. On the other hand, the further addition of $10\,\mu M$ Tef markedly overcame RAM-mediated reduction of exponential time constant of I_{Na} decaying by an increase in time constant to 99 ± 11 ms ($n = 8$, $p < 0.05$) (Figure 4C). Tef is known to activate I_{Na} as well as to slow the inactivation time course of the current.[38,48] Therefore, the experimental observations indicate that cell exposure to RAM results in an additional enhancement in the decaying of I_{Na} (a decrease in decaying time constant) elicited by a 1-s train of the depolarizing pulse.

3.5 | Effect of RAM on the recovery time course of I_{Na} inactivation after the conditioning train of depolarizing stimuli

A unique type of recovery from I_{Na} block as elicited by a train of conditioning depolarizing stimuli was demonstrated in previous studies.[43,49] Therefore, We continued
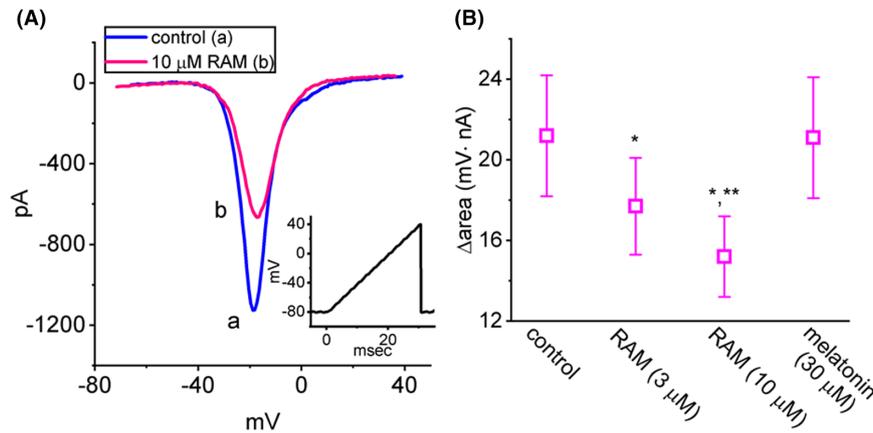
FIGURE 3 Modification by RAM of window I_{Na} ($I_{Na(W)}$) elicited by brief ascending ramp voltage (V_{ramp}). The current recordings were undertaken with the tested cell held at $-80\,mV$, and the V_{ramp} with a range between -80 and $+40\,mV$ was applied for a duration of 30 ms. (A) Representative current traces were obtained in the control period (a, blue color) and during the exposure to 10 μM RAM (b, pink color). Inset shows the voltage-clamp protocol applied, while the downward deflection displays the occurrence of inward current. (B) Summary scatter graph showing the effect of RAM (3 or 10 μM) and melatonin (30 mM) on the area (i.e., Δarea) of $I_{Na(W)}$ (mean ± SEM for each point; $n = 8$). The Δarea was measured at the voltages ranging between -40 and $+40\,mV$ during the ascending V_{ramp}. *Significantly different from control ($p < 0.05$) and **significantly different from RAM (3 μM) alone group ($p < 0.05$).

to investigate if such recovery of I_{Na} with or without the addition of RAM can occur. The voltage-clamp protocol used is illustrated in Figure 5A, the protocol consisted of the preceding conditioning train and a two-step voltage protocol, following the conditioning train. Different recovery patterns from slow inactivation in I_{Na} were found: the pattern obtained in response to the conditioning depolarizing stimuli emerged in a biphasic manner, while the pattern with no preceding conditioning train pulse was fit to a single exponential. As shown in Figure 5B, in the control period, experimental points fit well with the sum of two exponential functions, fast (τ_{fast}) and slow time constants (τ_{slow}). Also, the evolving values of both τ_{fast} and τ_{slow} rose upon the exposure to RAM. Table 1 summarizes the values used for estimating nonlinear recovery time course in the absence and presence of RAM (3 and 10 μM). The collective results suggested that the I_{Na} activated by a conditioning train of the pulse can shift a large fraction of Na_V channels to the slowly recovering pool in Neuro-2a cells. Moreover, RAM could make the fraction of slowly recovery pool of Na_V channels during the conditioning train pulse smaller, with an increase in both τ_{fast} and τ_{slow} values of recovery time course in these cells.

3.6 | The effect of RAM on delayed-rectifier K⁺ current ($I_{K(DR)}$) identified in Neuro-2a cells

We also investigated whether $I_{K(DR)}$ can be modified by the presence of RAM. $I_{K(DR)}$ was elicited by maintaining the tested cell at $-50\,mV$, and going through a series of

voltages ranging between -60 and $+50\,mV$ for a duration of 1 s as demonstrated in Figure 6A,B. Upon exposure to 3 μM RAM, the amplitude of $I_{K(DR)}$ measured at the voltages was substantially suppressed particularly above $-10\,mV$. The cells were stepped from -50 to $+40\,mV$ for 1 s, the measured $I_{K(DR)}$ decreased from 230 ± 21 to 79 ± 9 pA ($n = 8$, $p < 0.05$) after the addition of 3 μM RAM and returned to 226 ± 19 pA ($n = 8$) after washout of RAM.

We evaluated the relationship between RAM concentration and the $I_{K(DR)}$ amplitude in these cells by following a nonlinear least-squares fit to the experimental results (Figure 6C), the IC_{50} value of 2.9 μM was estimated to inhibit $I_{K(DR)}$ amplitude in Neuro-2a cells, and nearly complete inhibition of the current amplitude at a concentration of 100 μM of RAM was found.

3.7 | Effect of RAM on I_{Na} identified in pituitary tumor (GH₃) cells

Earlier studies have shown the ability of RAM to regulate prolactin secretion in pituitary cells.[50,51] Therefore, we further attempted to explore if the presence of RAM could modify I_{Na} in different types of excitable cells (e.g., GH₃ cells). As demonstrated in Figure 7A,B, as GH₃ cells were continually exposed to RAM (3 or 10 μM), the I_{Na} was considerably reduced. Also, the further addition of ProTx-II at a concentration of 1 μM, but still in the presence of RAM (10 μM), effectively suppressed I_{Na} amplitude(Figure 7B). The experimental results reflect that like the observations described above in Neuro-2a cells, the exposure to RAM can attenuate I_{Na} amplitude in these cells.
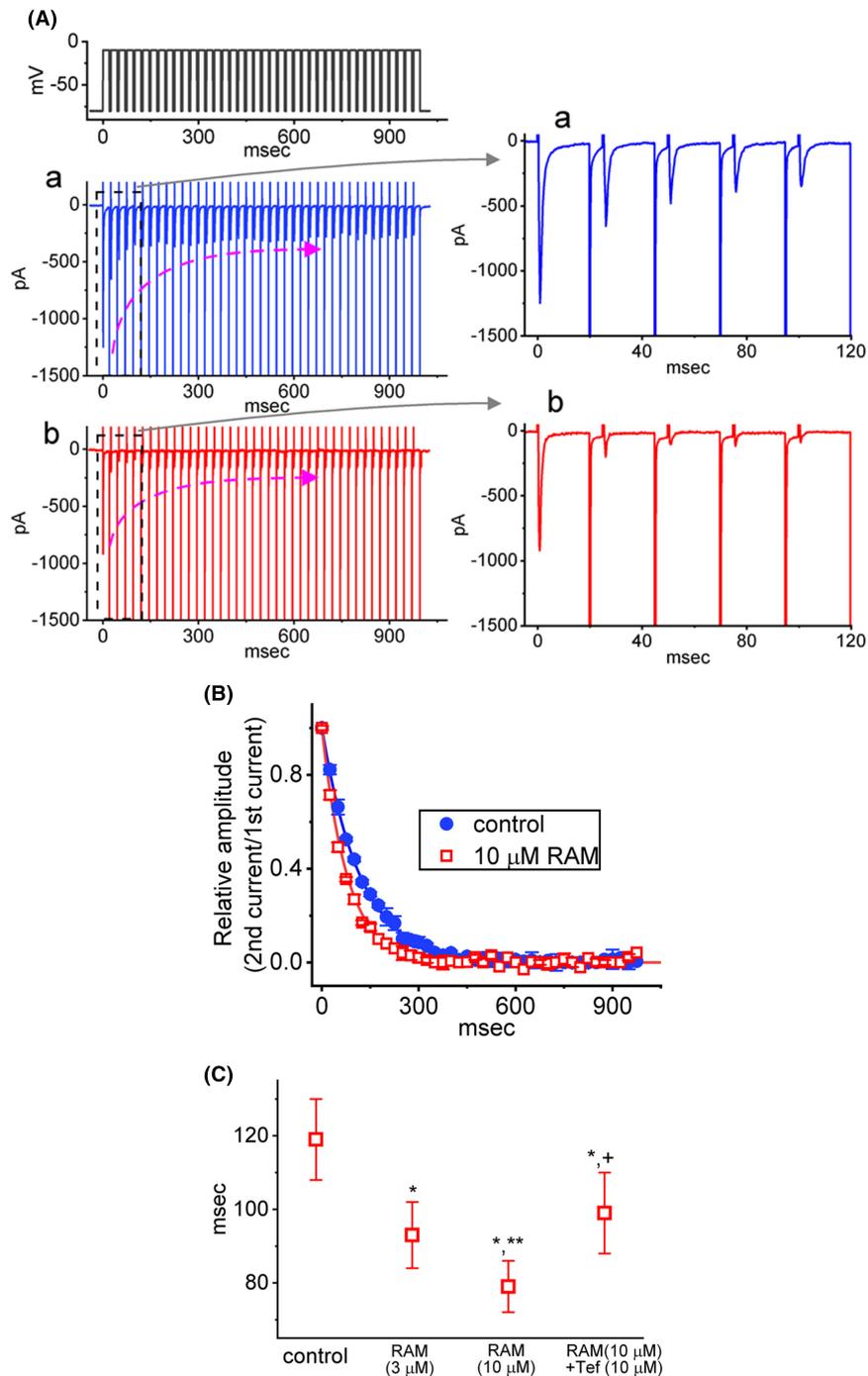
FIGURE 4 Effect of RAM on peak I_{Na} activated by a train of depolarizing pulses in Neuro-2a cells. The train that we applied to the tested cell was designed to comprise 40 20-ms pulses (stepped to −10 mV) separated by 5 ms intervals at −80 mV for a total duration of 1 s. (A) Representative current traces acquired in the control period (a, absence of RAM; blue color) and during the exposure to 10 μM RAM (b, red color). The top panel shows the voltage-clamp protocol applied. The dashed curved arrow in each panel denotes the decaying time course of peak I_{Na} during a train of depolarizing pulses. To provide a single I_{Na} trace, we expanded the traces inside dashed boxes at the left side to those at the right side. (B) The relationship of peak I_{Na} versus the pulse train duration acquired in the absence (●) and presence (□) of 10 μM RAM (mean ± SEM; $n = 8$ for each point). The continuous smooth lines over which the data points are overlaid are optimally fitted by a single exponential. Note that the addition of RAM can enhance the time course of I_{Na} decaying in response to a train of depolarizing pulses in an exponential fashion. (C) Summary scatter graph demonstrating effects of RAM (3 or 10 μM) and RAM (10 μM) plus tefluthrin (Tef, 10 μM) on the time constant of current decay activated by a train of depolarizing command voltage from −80 to −10 mV (mean ± SEM; $n = 8$ for each point). Each current amplitude was taken at the beginning of the depolarizing pulse. * Significantly different from control ($p < 0.05$), ** significantly different from RAM (3 μM) alone group ($p < 0.05$), and + significantly different from RAM (10 μM) alone group ($p < 0.05$).

3.8 | Inhibitory effect of RAM on spontaneous action potentials in GH$_3$ cells

In another set of experiments, we explored whether cell exposure to RAM could ater the spontaneous action potential in these cells. Under current-clamp conditions,
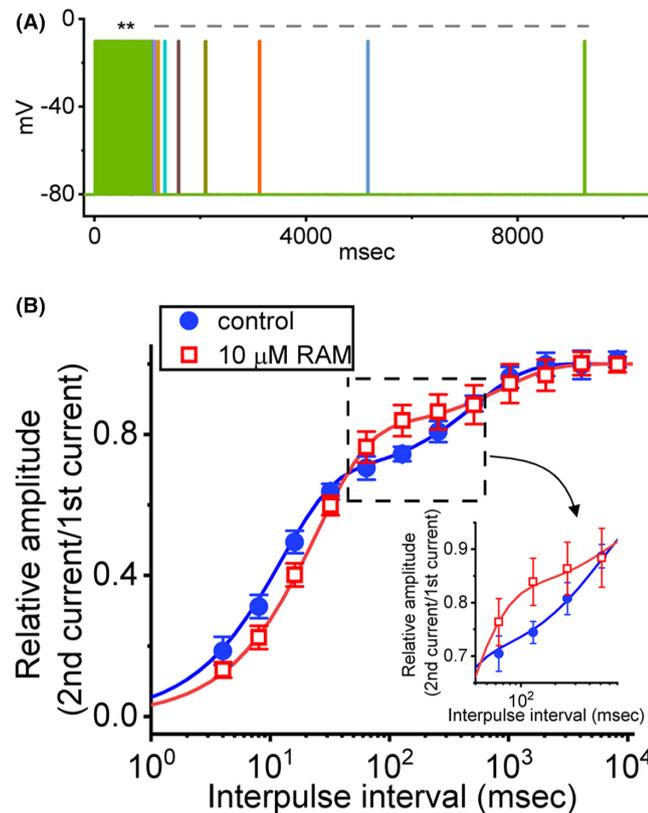

(A)

(B)

FIGURE 5 The recovery of I_{Na} inactivation during the train of conditioning depolarizing pulses acquired in the absence and presence of 10 μM RAM. Panel (A) shows the voltage-clamp protocol applied. Double asterisks show the train of conditioning depolarizing pulses from −80 to −10 mV, while horizontal dashed line indicates varying interpulse intervals with a geometric progression. (B) The relationships of the relative amplitude of peak I_{Na} versus the interpulse interval acquired in the absence (●) and presence (●) of 10 μM RAM (mean ± SEM; $n = 8$ for each point). The smooth curve acquired with or without the RAM addition was optimally fitted with a two-exponential function as elaborated in Section 2. Inset shown an expanded graph from the dashed box, indicating the presence of slowly recovering phase during I_{Na} recovery time course obtained with or without exposure to RAM. Note that the x-axis is illustrated with a logarithmic scale.

when GH$_3$ cells were exposed to RAM at a concentration of 3 or 10 μM, the firing frequency of action potentials was progressively decreased (Figure 8A,B). For example, the presence of 10 mM RAM resulted in a significant reduction of the firing frequency from 1.4 ± 0.7 to 9.2 ± 0.3 Hz ($n = 7$, $p < 0.05$). However, the resting membrane potential was unchanged in the presence of RAM. Furthermore, cell exposure to 30 μM melatonin alone did not change the firing frequency observed in these cells. It is therefore reasonable to assume that RAM-mediated decreased in the firing frequency is closely linked to its suppressive effect on I_{Na} presented above.

4 | DISCUSSION

In the current investigation, we found that RAM can attenuate I_{Na} in a concentration-dependent manner in Neuro-2a cells despite the unchanged overall steady-state I–V relation of peak I_{Na}. Furthermore, the addition of RAM can attenuate the magnitude (Δarea) of instantaneous $I_{Na (W)}$ elicited by short ascending V_{ramp}, whereas melatonin alone failed to affect $I_{Na(W)}$. During a train of depolarizing pulses, the decaying time constant of I_{Na} was considerably shortened by adding RAM. The recovery time constant (the τ_{fast} and τ_{slow} values) elicited by a conditioning train pulse arose during exposure to RAM. In addition, RAM effectively suppressed $I_{K (DR)}$ with an IC$_{50}$ value of 2.9 μM; and it could also attenuate the I_{Na} residing in pituitary GH$_3$ cells. Taken together, the present observations reveal that RAM can mediate changes in the magnitude and gating properties of I_{Na}. As previously well known, voltage-gated sodium channels initiate action potentials in brain neurons,[52] which are responsible for epileptogenicity (cortical neurons) and regulation of circadian rhythms (ventral suprachiasmatic nucleus neurons). A hypothesis could be made that the anti-seizure effects of RAM may be due to the I_{Na} attenuation in excitable cortical neurons and the restore of circadian rhythm could be a result of the decreased action potential firing rates in the circadian circuit according to current observation and previous literature.[53–55]

The time-dependent decline in I_{Na} during a 40-Hz train of depolarizing voltage commands (20 ms pulses applied from −80 to −10 mV at a rate of 40 Hz for a duration of 1 s)

	n	τ_{fast} (ms)	τ_{slow} (ms)	A	B
Control	8	12.1 ± 0.5	518 ± 12	0.68 ± 0.04	0.32 ± 0.02
RAM (3 μM)	8	18.3 ± 0.6*	762 ± 14*	0.78 ± 0.04*	0.22 ± 0.02*
RAM (10 μM)	8	21.5 ± 0.7*	882 ± 15*	0.81 ± 0.04*	0.19 ± 0.02*

Note: All values are mean ± SEM.

*Significantly different from controls ($p < 0.05$).

TABLE 1 Summary of data demonstrating the parameter values for effect of RAM on the recovery of I_{Na} block during the preceding train pulse. These parameters are elaborated in detail in Section 2.
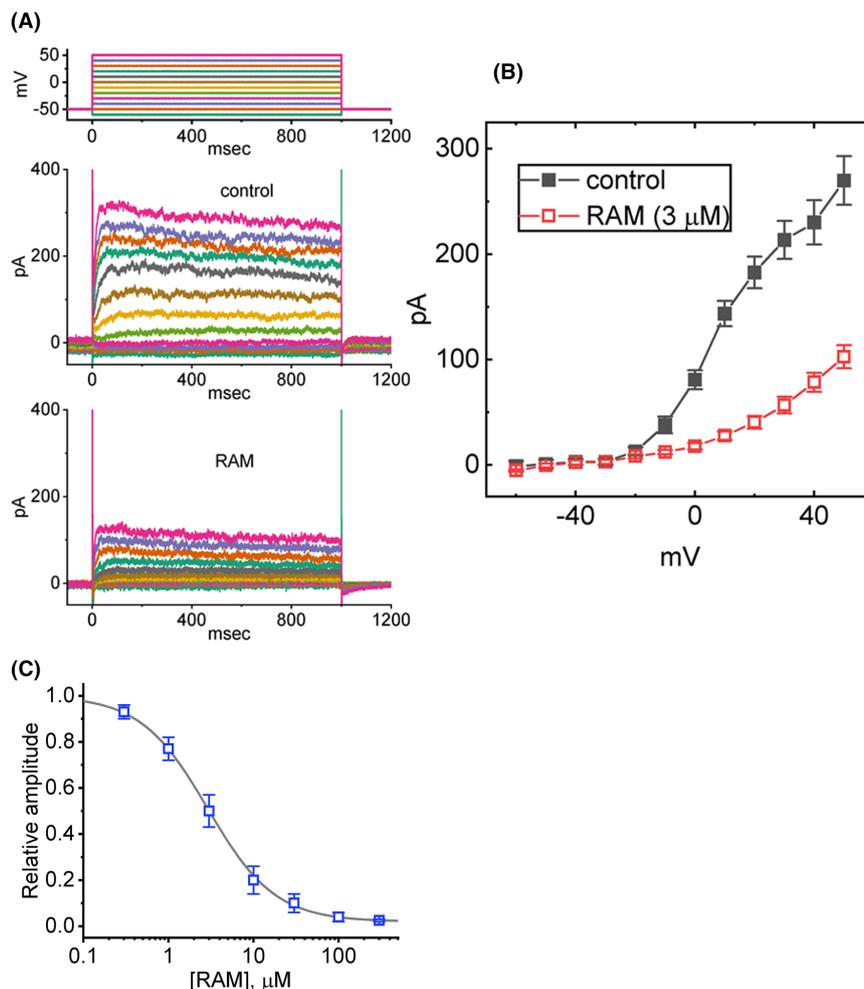
FIGURE 6 Effect of RAM on delayed-rectifier K$^+$ current ($I_{K(DR)}$) residing in Neuro-2a cells. This set of experiments was made in cells which were placed in Ca^{2+}-free, Tyrode's solution containing 1 μM TTX and CdCl$_2$, and we filled up the recording electrodes with K$^+$-enriched internal solution. (A) Representative current traces acquired in the control period (upper) and during cell exposure to 3 μM RAM. The top panel indicates the voltage-clamp protocol applied for the activation of $I_{K(DR)}$. (B) Average I–V relationship of $I_{K(DR)}$ acquired with or without the application of 3 μM RAM (mean ± SEM; $n = 8$). The $I_{K(DR)}$ amplitude was measured at the end-pulse of each command potential level. Note that cell exposure to RAM can depress $I_{K(DR)}$ amplitude particularly at the voltages above 0 mV. ■: control; □: in the presence of 3 μM RAM. (C) Concentration-dependent inhibition of RAM on $I_{K(DR)}$ amplitude (mean ± SEM; $n = 8$ for each point). Continuous gray line shows an overlay of the best fit to a modified Hill equation as described in Section 2. The IC$_{50}$ value needed for RAM-mediated block of $I_{K(DR)}$ in these cells was calculated as 2.9 μM.

was clearly observed in an exponential fashion as shown in Neuro-2a cells. Of additional interest, the exponential decrease in I_{Na} induced by challenging the tested cell with pulse train stimulation became pronounced during exposure to RAM. In the continued presence of RAM, further application of Tef could substantially reverse RAM-induced decrease in decaying time constant of I_{Na} during repetitive stimulations. It is thus possible from the present observations that the exposure to RAM would lead to a 'loss-of-function' change caused by a decrease in the decaying time constant of I_{Na} and that RAM-induced reduction of I_{Na} is intimately linked to the use-dependent decrease in I_{Na} during rapid repetitive stimuli or high-frequency firing.[43–45]

It is important to mention that Neuro-2a-cell exposure to RAM was able to alter the values of τ_{fast} and τ_{slow} of I_{Na} recovery elicited during the conditioning train pulse, as presented in Table 1. As such, upon the existence of RAM, the post-spike I_{Na} during rapid repetitive stimuli or high-frequency firing is closely associated with prolonged recovery of I_{Na} block. Given that recovery from current inactivation (i.e., fast and slow recovering phases existing in inactivating state) occurs through conformation changes of ion channels, it would be accompanied by attenuation in small residual steady Na$^+$ current in the presence of RAM. Meanwhile, cell exposure to RAM was noted to decrease the extent of $I_{Na(W)}$ in response to short ascending V$_{ramp}$, although melatonin itself had little or no effect on it. Unlike
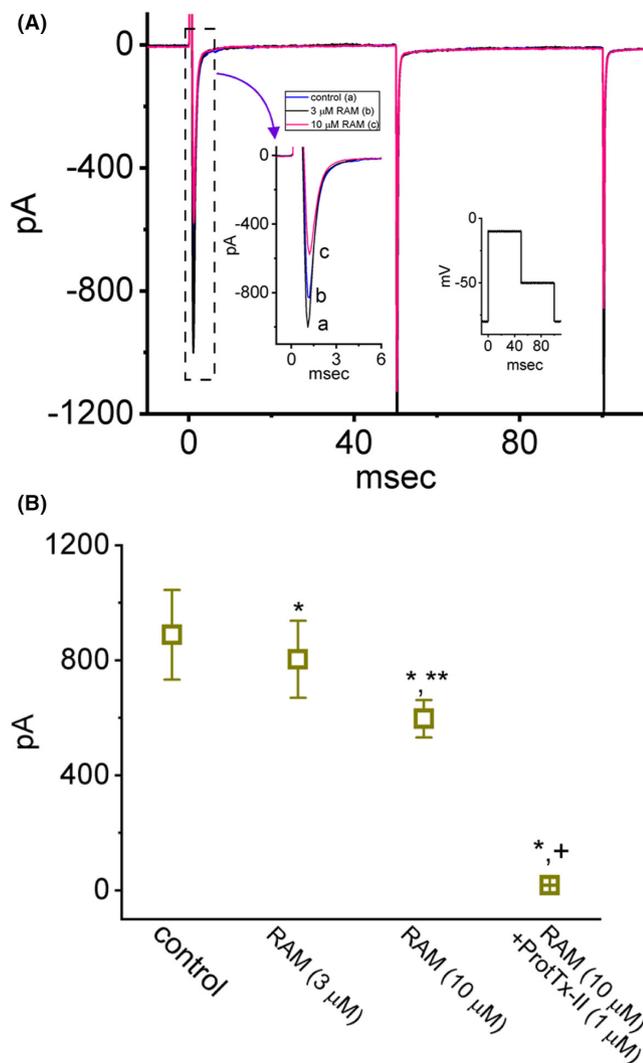
FIGURE 7 Effect of RAM on I_{Na} in pituitary tumor (GH₃) cells. The experiments were made as cells were bathed in Ca^{2+}-free, Tyrode's solution containing 10 mM TEA and 0.5 mM CdCl₂, while the recording electrode was filled up with Cs^+-enriched solution. (A) Representative current traces obtained in the control period (a, black color) and during exposure to 3 μM RAM (b, blue color) or 10 μM RAM (c, pink color). The left side in inset indicates the expanded record (i.e., magnified current traces) from the dashed box, while the right side is the voltage-clamp protocol applied. (B) Summary scatter graphs demonstrating effects of RAM (3 or 10 μM) and RAM (3 μM) plus ProTx II (1 μM) on peak I_{Na} in GH₃ cells (mean ± SEM; $n = 7$ for each point). Current amplitude (i.e., peak I_{Na}) was taken at the beginning of brief depolarizing command from −80 to −10 mV. *Signficantly different from control ($p < 0.05$), **significantly different from RAM (3 μM) alone group ($p < 0.05$), and + significantly different from RAM (10 μM) alone group ($p < 0.05$).

melatonin, RAM did not alert any other neurotransmitter system such as γ-aminobutyric acid (GABA) and serotonin.[56] Thus, our findings suggested that the effect of precluding the occurrence of subthreshold potential after the addition of RAM is expected partially related to diminished

magnitude of post-spike and steady currents on NaV channels, beside MT1 or MT2 receptors.[43,57]

The $I_{K (DR)}$ plays a significant role in regulating neuronal excitability, action potential duration, network synchronization, and may contribute to the pathophysiology of epileptic seizures.[11,21] Dysfunction in these channels can lead to hyperexcitability and aberrant neuronal firing, increasing the susceptibility to epileptic seizures.[12,13] Pharmacological modulation of delayed-rectifier K⁺ channels has been explored as a potential therapeutic strategy for managing epilepsy.[13,17] In this study, the presence of RAM was found to be effective at suppressing $I_{K (DR)}$ amplitude in Neuro-2a cells. It must be emphasized that the $I_{K(DR)}$ magnitude, particularly at the emergence of resurgent K⁺ tail currents during prolonged and rapid repetitive stimulation, can be attenuated in the RAM presence as a function of firing frequency.[12,13,16,58] The main reason is that upon RAM exposure, the availability of Na$_V$-channel openings during high-frequency firing would become further exacerbated, thus ultimately precluding the efficiency in synaptic transmission at high frequency.[20,46,59]

In light of the current investigations, besides the agonistic effects of RAM on MT₁ and MT₂ receptors as previously thought, our results suggest the direct inhibitory effects of RAM on the magnitude of I_{Na} and $I_{K (DR)}$ appear to be an obligate mechanism, which was not previously reported. Furthermore, RAM exerted an inhibitory effect on spontaneous action potentials in GH₃ cells, while melatonin alone had no effect on either the ion currents or action potentials in the excitable cells. It is therefore unlikely that RAM-induced change in ionic currents and membrane potential is dependent of its binding melatonin receptor(s).

According to earlier pharmacokinetic analyses, the plasma level of RAM administered with a single 16 mg of RAM in healthy volunteers was reported to reach 9–10 ng/mL (or around 0.04 μM).[60] This value tends to be lower than the IC₅₀ values required for RAM-mediated inhibition of membrane ionic currents presented here. However, it needs to be stressed that the responsiveness to RAM residing in excitable cells could heavily rely on different confounding variables, including the RAM concentration used, the pre-existing level of resting potential, varying discharge patterns of spike firing (particularly at high-frequency firing), and in any combinations of the three. Moreover, owing to its lipophilicity, the RAM molecule is believed to access the cell easily, and it can readily penetrate the blood–brain barrier.[61–65] Following the administration, it might be deposited in different tissues at a higher concentration and/or over a longer duration of accumulation (e.g., adipose or neural tissues and oral cavity).[65–67]

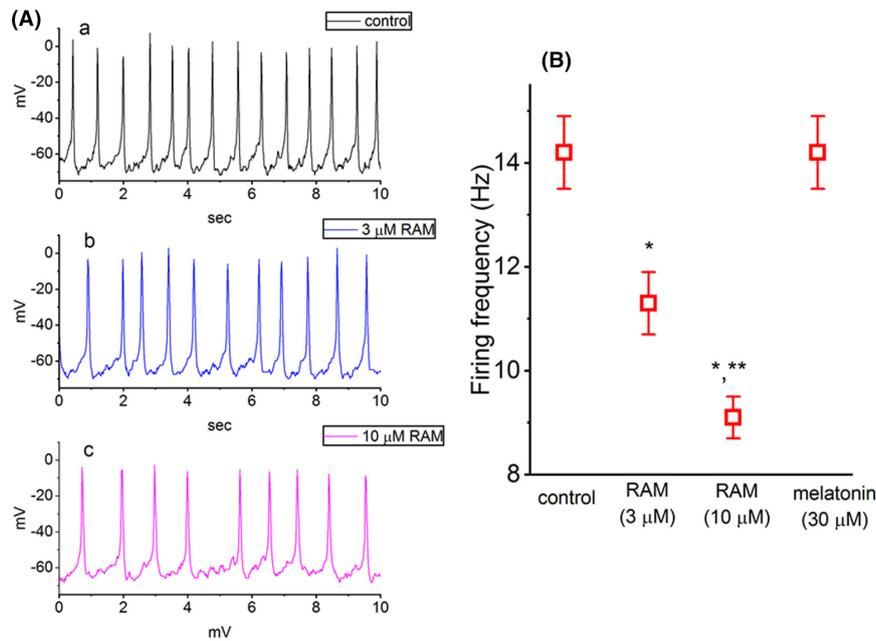Finally, we conducted another series of experiments showing that GH3-cells exposure to RAM decreased the

FIGURE 8 Suppressive effect of RAM on spontaneous action potentials measured from GH_3 cells. These whole-cell potential recordings were conducted in cells bathed in normal Tyrode's solution, and the examined cells were held in current-clamp mode with a hoding ucrrent of 0 nA. (A) Representative potential traces shown in the absence (a) and presence of 3 mM RAM (b) or 10 mM RAM (c). (B) Summary scatter graph demonstrating effect of RAM (3 or 10 μM) and melatonin (30 μM) on the firing frequency of spontaneous action potentials (mean ± SEM; $n = 7$ for each point). *Signficantly different from control ($p < 0.05$), and ** significantly different from RAM (3 μM) alone group ($p < 0.05$).

firing frequency of spontaneous action potentials observed under current-clamp conditions. Consequently, RAM-mediated effect on INa did not appear to be non-specific and closely associated with its ability to decrease spontaneous action potentials. Taken together, to what extent the perturbations by RAM on plasmalemmal ionic currents are involved in any changes in functional activities of different excitable cells still warrants further investigations. It may explain the applicability of RAM for the improvement of epilepsy.

AUTHOR CONTRIBUTIONS

Conceptualization: P.-M.W, Y.-F.T, S.-N.W. *Methodology*: M.-C.Y, H.-Y.C, S.-N.W.; *Software*: M.-C.Y, Y.-H.W, S.-N.W.; *Validation*: P.-M.W, Y.-F.T, H.-Y.C., and S.-N.W.; formal analysis, M.-C.Y, S.-N.W.; *Investigation*: P.-M.W, H.-Y.C., and S.-N.W.; *Resources*: M.-C.Y, Y.-H.W, S.-N.W.; *Data curation*: S.-N.W.; *Writing—original draft preparation*: P.-M.W.; *Writing—review and editing*: Y.-F.T, S.-N.W.; *Visualization*: H.-Y.C., and S.-N.W.; *Supervision*: S.-N.W.; *Project administration*: Y.-F.T., S.-N.W.; *Funding acquisition*: P.-M.W, Y.-F.T. All authors have read and agreed to the published version of the manuscript.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

DISCLOSURES

All the authors report no declarations of interest that are directly relevant to this study. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

ETHICS STATEMENT

Ethical approval is not applicable for this article.

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