Activity-dependent modulation of neuronal \(K_V\) channels by retinoic acid enhances \(Ca_V\) channel activity

The metabolite of vitamin A, retinoic acid (RA), is known to affect synaptic plasticity in the nervous system and to play an important role in learning and memory. A ubiquitous mechanism by which neuronal plasticity develops in the nervous system is through modulation of voltage-gated \(Ca^{2+}\) (\(Ca_V\)) and voltage-gated \(K^+\) channels. However, how retinoids might regulate the activity of these channels has not been determined. Here, we show that RA modulates neuronal firing by inducing spike broadening and complex spiking in a dose-dependent manner in peptidergic and dopaminergic cell types. Using patch-clamp electrophysiology, we show that RA-induced complex spiking is activity dependent and involves enhanced inactivation of delayed rectifier voltage-gated \(K^+\) channels. The prolonged depolarizations observed during RA-modulated spiking lead to an increase in \(Ca^{2+}\) influx through \(Ca_V\) channels, though we also show an opposing effect of RA on the same neurons to inhibit \(Ca^{2+}\) influx. At physiological levels of \(Ca^{2+}\), this inhibition is specific to \(Ca_V\) (not \(Ca_A\)1) channels. Examining the interaction between the spike-modulating effects of RA and its inhibition of \(Ca_V\) channels, we found that inhibition of \(Ca_V\) channels limits the \(Ca^{2+}\) influx resulting from spike modulation. Our data thus provide novel evidence to suggest that retinoid signaling affects both delayed rectifier \(K^+\) channels and \(Ca_V\) channels to fine-tune \(Ca^{2+}\) influx through \(Ca_V\)2 channels. As these channels play important roles in synaptic function, we propose that these modulatory effects of retinoids likely contribute to synaptic plasticity in the nervous system.

Neural circuits are able to process and store information largely as a result of their ability to undergo plasticity and modulation. A ubiquitous mechanism through which neuronal plasticity occurs is through modulation of voltage-gated \(Ca^{2+}\) (\(Ca_V\)) and voltage-gated \(K^+\) (\(K_V\)) channels (1, 2). \(Ca_V\) channel activity influences synaptic transmission and gene transcription, whereas \(K_V\) channel activity regulates action potential repolarization and neuronal excitability. Neurons can possess three major subtypes of \(Ca_V\) channels, \(Ca_V\)1, \(Ca_V\)2, and \(Ca_V\)3. The \(Ca_V\)1 (L-type) channels are activated at high voltages, are largely present in neuronal dendrites and the cell body, and mediate activity-dependent gene transcription (3, 4). \(Ca_V\)2 (non-L-type) channels also activate at high voltages, are largely present at presynaptic terminals and mediate synaptic transmission, though can also mediate \(Ca^{2+}\) signaling in neuronal cell bodies (1, 4). Finally, \(Ca_V\)3 (T-type) channels activate at low voltages and shape the firing properties of neurons (5). There are many different gene families for \(K_V\) channels. Two broad categories of \(K_V\) channels are the rapidly inactivating A-type channels and the nonrapidly inactivating delayed rectifier channels (6). Both these categories of \(K_V\) channels have been implicated in shaping the firing properties of neurons, (e.g., hippocampal neurons) and have thus been implicated in synaptic plasticity in the nervous system (2, 7).

Retinoic acid (RA), the active metabolite of vitamin A, has recently emerged as a critical regulator of neural plasticity in the adult brain (in addition to its important roles in nervous system development). It can exert both genomic and non-genomic effects by binding to retinoic acid receptors and retinoid X receptors (RXR) (8, 9), though its ability to bind directly to other signaling molecules has also been shown (10). RA signaling plays an important role in spatial learning and memory in rodents (11–13), and at the cellular level, it contributes to long-term potentiation (LTP) and/or long-term depression (LTD) in the hippocampus (11, 13, 14). RA signaling can also interact with \(Ca^{2+}\) signaling during homeostatic plasticity at excitatory synapses (8, 15–17). In particular, activation of RA signaling induces local synthesis and membrane insertion of glutamate receptors, subsequently increasing \(Ca^{2+}\) influx into the postsynaptic neurons in an activity-dependent manner (8, 15–17). RA has also been shown to reduce inhibitory synaptic transmission through rapid endocytosis of postsynaptic gamma-aminobutyric acid type A receptors (18). In addition to these postsynaptic effects of RA, it can both increase or decrease inhibitory synaptic transmission through presynaptic mechanisms, in a cell type-specific manner (19, 20). Thus, RA signaling can regulate activity-dependent plasticity at different synapses through diverse presynaptic and postsynaptic mechanisms.

In addition to modulating synaptic efficacy in the vertebrate central nervous system (CNS), RA signaling also affects neural circuitry in invertebrates. We have previously shown that RA is required for normal associative learning and memory in the mollusc, *Lymnaea stagnalis* (21), and can affect neuronal synapse formation and modulation *in vitro* (22). However, the activity-dependent mechanisms by which RA regulates neural plasticity are largely unknown. In *Lymnaea* neurons, RA

* For correspondence: Gaynor E. Spencer, gspencer@brocku.ca.
produces spike broadening and complex spiking, which includes burst firing and plateau potentials (23). Such a modulation of spike shape and firing patterns is known to play a role in synaptic plasticity (during learning and memory) and can result in enhanced synaptic transmission in neural circuits (24). We have also shown that RA signaling inhibits CaV channels in Lymnaea neurons (25). Whether the RA-induced spike broadening and complex spiking is activity dependent (and thus contributes to activity-dependent processes such as learning and memory), and how this effect interacts with concurrent RA-mediated inhibition of CaV channels, is not yet known. In this study, we examine this interaction and also determine whether changes in Kv channel function contribute to these modulatory effects of RA on spike broadening and complex spiking activity.

We show that micromolar concentrations of RA (similar to those eliciting effects in mouse and human neurons) produce spike broadening and complex spiking in an activity-dependent manner and in diverse neuronal cell types. By playing back neuronal firing patterns (action potential clamp) into single neurons in vitro, we show that RA-mediated inhibition of CaV2 channels limits the enhanced Ca2+ influx that occurs during RA-mediated spike broadening and activity-dependent complex spiking. We also determine that spike broadening and activity-dependent complex spiking likely occurs as a result of RA-mediated inhibition of delayed rectifier Kv channels. These data provide novel insights into how multiple forms of neuromodulation mediated by RA might tune Ca2+ influx (and thus potentially neurotransmitter release) at neuronal synapses.

Results

RA-induced neuromodulation is both concentration and activity dependent

Spike broadening and burst firing are important for information transfer and plasticity in neural circuits. We have previously shown that all-trans RA produces spike broadening and complex spiking (burst firing and plateau potentials) in Lymnaea visceral F (VF) neurons (23). However, the activity-dependence of these effects, as well as the underlying cellular mechanisms, has not yet been determined.

VF neurons were incubated overnight in varying concentrations of RA (ranging from 1 to 5 μM) or the equivalent concentration of dimethyl sulfoxide (DMSO) (as controls). The following day, individual neurons were current clamped and stimulated using a current-step protocol ranging from −100 to 400 pA in intervals of 25 pA. To determine whether RA-mediated spike broadening and complex spiking was activity dependent, we analyzed spike half-width (spike broadening) and complex spiking at the first current step that elicited a minimum of three action potentials (rheobase 1) and at the three subsequent depolarizing current injection steps (rheobases 2–4). We found that all concentrations of RA between 1 and 5 μM produced spike broadening (representative example is shown in Fig. 1A). We compared the spike half-width in the presence or the absence of RA at all four rheobases and for each concentration of RA. A two-way ANOVA of spike half-width at each concentration (1, 2, 2.5, 3, and 5 μM RA) revealed a significant effect of treatment (RA), but not rheobase (activity), for all concentrations including the lowest concentrations of 1 μM (F(1,84) = 11.546; p = 0.001; Fig. 1B) and 2 μM (F(1,99) = 13.173; p < 0.001; Fig. 1C).

We also determined whether RA influenced other physiological properties of neuronal activity. A two-way ANOVA of the peak voltage of the action potential across rheobases 1 to 4, for each concentration of RA, revealed that all concentrations of RA (except 1 μM) significantly reduced the action potential peak voltage, including the lower concentration of 2 μM (F(1,99) = 5.175; p = 0.025; RA: 39.24 ± 9.7 mV; DMSO: 41.9 ± 5.7 mV). Similarly, all concentrations of RA (including the lowest concentration of 1 μM [F(1,84) = 74.152; p < 0.001]) significantly depolarized the peak voltage of the afterhyperpolarization (AHP) (1 μM RA: −48.93 ± 6.4 mV; DMSO: −58.91 ± 5.8 mV), indicative of a reduced AHP amplitude. However, there were minimal effects of RA on the resting membrane potential (RMP) or input resistance; only 2.5 μM RA had a significant effect on the RMP (RA: −52.77 ± 7.1 mV; DMSO: −44.25 ± 7.7 mV; t = −2.572; p = 0.019), whereas only 3 μM significantly reduced the input resistance (RA: 661.24 ± 275.5 MΩ; DMSO: 919.86 ± 273.2 MΩ; t = −2.137; p = 0.046).

To determine the concentration dependence of complex spiking induced by RA, statistical analysis (Fisher’s exact tests) revealed that all concentrations of RA produced a significant increase in the proportion of cells exhibiting complex spiking (1 μM RA: 69%; p = 0.002; 2 μM RA: 81%; p < 0.001; 2.5 μM RA: 82%; p < 0.001; 3 μM RA: 75%; p = 0.001; 5 μM RA: 86%; p < 0.001) compared with the equivalent concentrations of DMSO, at which no cells (0%) exhibited complex spiking. These data suggest that concentrations of RA in a range similar to those previously estimated to occur in the Lymnaea CNS (26), and similar to those that induce effects in vertebrate neurons, produce both spike broadening, changes in spike amplitude and AHP, as well as complex firing in individual VF neurons.

To determine whether the effect of RA on complex spiking was activity dependent, we compared the number of complex spikes that occurred across rheobases 1 to 4, for each RA concentration. An example of complex spikes in the presence of 1 μM RA (at rheobase 4) is shown in Figure 2A. These representative recordings in Figure 2A also illustrate how the number of elicited spikes significantly increased from rheobase 1 to 4 (1 μM RA: H = 8.827; p = 0.032, ANOVA on ranks). This was also true for 2, 3, and 5 μM RA (and DMSO), confirming an activity-dependent increase in spike number across rheobases. A one-way ANOVA on ranks revealed that the incidence of complex spiking was found to be activity dependent at lower concentrations of 1 μM RA (H = 8.177; p = 0.042; Fig. 2B), 2 μM RA (H = 9.019; p = 0.029; Fig. 2C), and 3 μM RA (H = 9.138; p = 0.028; Fig. 2D), but not at the higher concentration of 5 μM (H = 1.003; p = 0.8; Fig. 2E). A concentration of 2 μM RA, which elicits both spike broadening and activity-dependent complex spiking, was chosen for use throughout the remainder of this study.
RA-induced neuromodulation occurs across multiple cell types

We next determined whether RA would produce spike broadening and activity-dependent complex spiking in other neuronal cell types, or whether this was specific to VF neurons. We assessed the effects of RA on the large dopaminergic interneuron, right pedal dorsal 1 (RPeD1, n = 7), the neuroendocrine right parietal B (RPB) cells (n = 8), or the right parietal A (RPA) respiratory motor neurons (n = 6). A two-way ANOVA of spike half-width revealed a significant effect of treatment on RPeD1 ($F_{(1,48)} = 61.375; p < 0.001$), RPB ($F_{(1,54)} = 12.363; p < 0.001$), and RPA cells ($F_{(1,40)} = 16.601; p < 0.001$), indicating that RA significantly increased spike half-width in all three cell types (compared with DMSO). RA also significantly increased the proportion of cells exhibiting complex spiking at any of the four rheobases (RPeD1: RA 71.4%, DMSO 0%; $p = 0.021$; RPB: RA 100%, DMSO 0%; $p < 0.001$; RPA: RA 83.3%, DMSO 0%; $p = 0.015$; Fisher’s exact tests). The ability of RA to induce complex spiking was also activity dependent in RPeD1 (H = 9.998; $p = 0.019$; Fig. 2F) and RPB cells (H = 16.395; $p < 0.001$; Fig. 2G) but not in RPA cells ($F_{(3,20)} = 1.114; p = 0.367$). These data suggest that RA induces activity-dependent complex spiking in diverse neuronal subtypes, including dopaminergic and peptidergic cells, suggesting a possible wide range of influence on distinct cell types within the brain.

RA modulates Ca$_{V}$2 channels in physiological [Ca$^{2+}$]$_{e}$

As Ca$_{V}$ channels play an ubiquitous role in synaptic transmission and activity-dependent changes in gene expression, modulation of their activity might directly influence activity-dependent changes in neural circuits. We have previously shown that RA (1 and 5 μM) inhibits Ca$_{V}$ channels by shifting the voltage dependence of channel activation; a biophysical property that determines at which voltage the channels open, thus determining their activity during neuronal firing. However, the previous study was performed using 10 mM barium as the charge carrier, eliminating any subsequent effects of calcium influx (25). As RA is known to interact with Ca$^{2+}$ signaling at hippocampal synapses (17, 27), we deemed it necessary to examine the effects of RA on Ca$_{V}$ channels in the presence of physiological concentrations of extracellular Ca$^{2+}$.

VF neurons were cultured overnight in the presence or the absence of RA and then voltage clamped in the presence of extracellular Ca$^{2+}$ (4.1 mM). Cells were held at −115 mV for 1 s and then stepped to potentials between −115 mV and +55 mV for 400 ms, in 5 mV increments. Raw recordings in Figure 3A illustrate the reduction in I$_{Ca}$ in the presence of 2 μM RA. A two-way ANOVA of peak current density revealed a significant interaction between treatment and voltage ($F_{(34,1260)} = 2.758; p < 0.001$). The current–voltage (IV)
relationship in Figure 3B illustrates that RA inhibited ICa at potentials between −15 and +35 mV. However, RA did not significantly affect the voltage dependence of channel activation (Fig. 3C), the voltage of half-maximal activation (RA: −2.683 ± 7.1 mV; DMSO: −5.224 ± 6.4 mV; p = 0.260), or the slope factor (RA: 5.777 ± 1.9; DMSO: 5.179 ± 1.6; p = 0.186).

Two additional properties that influence Ca2+ influx through CaV channels are the voltage dependence of inactivation (the inactivation that occurs at a particular membrane potential) and the recovery from inactivation (the time dependence of the removal of inactivation at negative membrane potentials). However, a two-way ANOVA of ICa inactivation revealed that treatment (RA versus DMSO) had no
effect on the voltage dependence of inactivation ($F_{(1,490)} = 1.252; p = 0.264$). RA had no effect on the voltage of half-maximal inactivation (RA: $-14.362 \pm 4.5$ mV; DMSO: $-12.091 \pm 4.4$ mV; $t = -1.021; p = 0.324$) or the slope of inactivation (RA: $4.88 \pm 0.4$; DMSO: $4.504 \pm 0.77; t = 1.313; p = 0.210$). A two-way ANOVA of recovery from inactivation also revealed no significant effect of treatment ($F_{(1,294)} = 0.242; p = 0.623$), suggesting that RA had no effect on the recovery from inactivation of CaV channels. Though it was not possible to record firing activity and Ca$^{2+}$ currents in the same cell (because of the presence of K$^+$ channel blockers), overall these data indicate that concentrations of RA that produced activity-dependent complex spiking also inhibited $I_{Ca}$ in the same cell type.

RA specifically inhibits CaV2 channels in physiological [Ca$^{2+}$]

We next determined, using physiological concentrations of Ca$^{2+}$, whether RA specifically affected L-type or non–L-type channels. VF neurons were cultured in the presence or the absence of RA overnight and then voltage clamped to record $I_{Ca}$. The Ca$^{2+}$1 (L-type) channel blocker, nifedipine (Sigma–Aldrich; 10 $\mu$M), was utilized to separate Ca$^{2+}$1 (non–L-type) currents from Ca$^{2+}$1 (L-type) currents (28, 29). The proportional block of $I_{Ca}$ by nifedipine indicated that Ca$^{2+}$1 current comprised approximately 20 to 25% of total $I_{Ca}$. The proportional block by nifedipine was slightly (but nonsignificantly) greater following RA treatment, compared with DMSO (RA: 26.839% $\pm$ 15.7; DMSO: 20.26% $\pm$ 12.1; $t = 1.008; p = 0.329$).

A two-way ANOVA of nifedipine-insensitive (Ca$^{2+}$1; non–L-type) $I_{Ca}$ revealed a significant interaction between treatment (RA versus DMSO) and voltage ($F_{(34,665)} = 2.244; p < 0.001$). Raw recordings (Fig. 4A) and the IV relationship (Fig. 4B) illustrate the inhibition of Ca$^{2+}$2 channels by RA at voltages ranging from $-10$ to $+20$ mV. Furthermore, the activation curve in Figure 4C illustrates that RA inhibited Ca$^{2+}$2 by shifting the voltage dependence of channel activation to more positive potentials; RA significantly increased the voltage of half-maximal activation (Fig. 4D) but had no significant effect on slope factor (RA: $7.101 \pm 1.6$; DMSO: $6.533 \pm 1.3; t = 0.906; p = 0.376$). In contrast, RA had no significant effect on Ca$^{2+}$1 channels (Fig. 4, E and F). These data suggest that concentrations of RA (2 $\mu$M), known to produce activity-dependent complex spiking in these cells, also inhibited Ca$^{2+}$2 (but not Ca$^{2+}$1) channels, by shifting the voltage dependence of channel activation to more positive potentials.

RA-mediated inhibition of Ca$^{2+}$1 channels limits Ca$^{2+}$ entry during activity-dependent neuromodulation

The aforementioned results indicate that the RA-mediated spike broadening and activity-dependent complex spiking occurs in the same cell type and at the same concentrations as the RA-mediated inhibition of Ca$^{2+}$2 channels. Presumably, the prolonged depolarizations occurring during spike broadening and complex spiking would enhance Ca$^{2+}$ influx through Ca$^{2+}$ channels, and it is possible that, if occurring concurrently, RA might induce opposing effects on Ca$^{2+}$ signaling. We next determined whether spike broadening and activity-dependent complex spiking does indeed enhance Ca$^{2+}$ influx, and if so, whether this is influenced by concurrent RA-induced inhibition of Ca$^{2+}$2 channels. To this end, we utilized the action potential clamp technique, which uses previously recorded neuronal activity (spiking) as the voltage stimulus for recording ion channel activity. Specifically, the voltage stimulus protocol consisted of a total of nine action potentials...
obtained from firing activity (rheobase 4) in a cell exposed to either 2 μM RA or 0.02% DMSO. The voltage protocol from DMSO treatment will be referred to hereafter as the "control" voltage protocol, whereas that from the RA-treated cell (exhibiting spike broadening and complex spiking) will be referred to as the "modulated" voltage protocol.

VF cells were again cultured overnight in the presence or the absence of RA. Each cell was held at −115 mV for 5 s, stepped to −55 mV (approximate RMP) for 5 s, followed by the "control" and then the "modulated" voltage protocols (Fig. 5A).

We analyzed current area density (total I_{Ca}) generated by each spike from both control and modulated voltage protocols and in cells treated with either RA or DMSO (representative traces in Fig. 5B). The modulated voltage protocol significantly increased I_{Ca} during each spike (compared with the control protocol), suggesting that the spike broadening and complex spiking induced by 2 μM RA does in fact increase Ca^{2+} influx through Ca_{V} channels. This spike broadening–induced increase in I_{Ca} from the modulated voltage protocol occurred in cells exposed to either RA (red circles) or DMSO (green triangles) (Fig. 5C; # symbols), though was significantly enhanced in DMSO-treated cells compared with RA-treated cells.

Figure 4. RA inhibits Ca_{V}2 channels by shifting the voltage dependence of channel activation. A, raw representative recordings (obtained at +5 mV) illustrating the reduced amplitude of I_{Ca} through Ca_{V}2 channels following exposure to 2 μM RA (red, lower trace) compared with 0.02% DMSO (black, upper trace). B, IV relationship showing that 2 μM RA (red circles) inhibited the peak current density through Ca_{V}2 channels at potentials from −10 to +20 mV. C, activation curve illustrating that RA shifted the voltage dependence of channel activation of Ca_{V}2 channels to more positive potentials. D, RA significantly increased the voltage of half-maximal activation, compared with DMSO. Bracketed values represent n values. *p ≤ 0.05; **p ≤ 0.01; and ***p ≤ 0.001 (compared with DMSO). Error bars represent SD. E, raw representative recordings (obtained at +5 mV) showing no difference in Ca_{V}1 channel currents following exposure to either 2 μM RA (red, lower trace) or to 0.02% DMSO (black, upper trace). F, IV relationship showing that 2 μM RA (red circles) had no significant effect on peak current density of Ca_{V}1 channels compared with DMSO (black squares). Ca_{V}, voltage-gated Ca^{2+} channel; DMSO, dimethyl sulfoxide; I_{Ca}, calcium current; IV, current–voltage; RA, retinoic acid.
Figure 5. Spike broadening and complex spiking enhances Ca\textsuperscript{2+} influx via Ca\textsubscript{v} channels, but this is limited by concurrent exposure to RA. A, illustrations of the control and modulated voltage protocols. The control protocol consists of nine action potentials recorded from a DMSO-exposed cell (at rheobase 4), whereas the modulated protocol consists of nine action potentials recorded from an RA-exposed cell. B, raw recordings of I\textsubscript{Ca} (top traces) corresponding to the nine action potentials comprising the control and modulated voltage protocols (bottom traces), in the presence of either 0.02% DMSO (black, left) or 2 μM RA (red, right). C, the modulated voltage protocol significantly enhanced I\textsubscript{Ca} (in cells exposed to both RA [red, n = 11] or DMSO [green, n = 9]), compared with the control voltage protocols (in cells exposed to either RA [blue, n = 11] or DMSO [black, n = 9]). # represents the comparison between modulated and control protocols (*p \leq 0.05; **p \leq 0.01; and ***p \leq 0.001). The modulated protocol applied to cells exposed to DMSO showed enhanced I\textsubscript{Ca} compared with the modulated protocol in cells exposed to RA. * represents the comparison between modulated protocols in RA (red circles) and DMSO (green triangles) (*p \leq 0.05; **p \leq 0.01). ! represents the enhanced I\textsubscript{Ca} between the modulated protocol in RA (red circles) and the control protocol in DMSO (black squares) (tp \leq 0.05; !tp \leq 0.01; and !!tp \leq 0.001). Ca\textsubscript{v}, voltage-gated Ca\textsuperscript{2+} channel; DMSO, dimethyl sulfoxide; I\textsubscript{Ca}, calcium current; RA, retinoic acid.
Ion channel neuromodulation by retinoic acid

It is clear that during concurrent spike broadening and exposure to RA, the IC$_{a}$-enhancing effects of RA predominate. A comparison of IC$_{a}$ from the modulated protocol in RA-exposed cells (red circles), to IC$_{a}$ from the control protocol in DMSO-exposed cells (black squares), indeed produced a significant interaction of treatment and spike number ($F_{(2,160)} = 4.2; p < 0.001$) with enhanced IC$_{a}$ in spikes 2 to 9 of RA-treated cells (Fig. 5C; * symbols). Thus, the inhibition of IC$_{a}$ (through CaV$_{2}$ channels) by RA limited (but did not prevent) the increase in IC$_{a}$ that occurred during prolonged depolarizations and neuronal activity (such as during spike broadening and complex spiking).

RA limits Ca$^{2+}$ influx through CaV$_{2}$ channels during neuronal activity

CaV$_{1}$ channels, predominately localized to dendrites and cell bodies (where they play a primary role in activity-dependent gene regulation), appear to be unaffected by RA in physiological Ca$^{2+}$. In contrast, CaV$_{2}$ channels, predominately localized to presynaptic terminals (where they mediate neurotransmitter release), are inhibited by RA. Therefore, RA-mediated spike broadening and complex spiking, which occur in both cell bodies and isolated neurites (23), may have compartment-specific effects because of different localizations of the Ca$^{2+}$ channel subtypes. We next determined whether the enhanced Ca$^{2+}$ influx occurring during spike modulation occurs via CaV$_{1}$ and/or CaV$_{2}$ channels.

CaV$_{1}$ and CaV$_{2}$ currents were isolated and analyzed following application of both control and modulated voltage protocols. In cells exposed to either RA or DMSO, the modulated voltage protocol enhanced IC$_{a}$ through CaV$_{2}$ during all spikes, compared with the control voltage protocol (Fig. 6, A and B; # symbols), confirming that spike broadening and complex spiking enhanced Ca$^{2+}$ influx through CaV$_{2}$ channels.

Once again however, this increase in IC$_{a}$ through CaV$_{2}$ was significantly greater following DMSO exposure, compared with RA exposure (Fig. 6B; * symbols). These data suggest that spike broadening and activity-dependent complex spiking enhances IC$_{a}$ through CaV$_{2}$ and that RA-mediated inhibition of CaV$_{2}$ limits (but does not prevent) this enhancement. When we again compared CaV$_{2}$ IC$_{a}$ following the modulated voltage protocol in cells exposed to RA (red circles), with the control voltage protocol in DMSO-exposed cells (black squares), there was a significant effect of treatment (two-way ANOVA [$F_{(1,171)} = 26.156; p < 0.001$]). However, in this instance, IC$_{a}$ was only enhanced during the complex spikes (spikes 6, 7, and 9; Fig. 6B; ! symbols) but not during the broadened spikes (spikes 1–5).

Analysis of CaV$_{1}$ currents revealed that the modulated voltage protocol enhanced CaV$_{1}$ IC$_{a}$, compared with the control voltage protocol at spikes 2 to 9 (Fig. 6C; # symbols) for both RA- and DMSO-treated cells. However, treatment with RA had no significant effect on CaV$_{1}$ IC$_{a}$ during the modulated protocols (compared with treatment with DMSO). This suggests that the spike broadening and activity-dependent complex spiking can enhance overall Ca$^{2+}$ signaling through CaV$_{1}$ channels regardless of concurrent exposure to RA, because unlike CaV$_{2}$ channels, CaV$_{1}$ channels are not inhibited by RA in physiological Ca$^{2+}$.

In summary, these data show that activity-dependent spike broadening and complex spiking (such as that induced by RA) results in the enhancement of IC$_{a}$ through both CaV$_{1}$ and CaV$_{2}$ channels. However, exposure to RA also inhibits CaV$_{2}$ channels (but not CaV$_{1}$). Though spike broadening is sufficient to compensate for this inhibition, CaV$_{2}$ IC$_{a}$ is only enhanced overall during complex spiking and not during spike broadening.

RA inhibits delayed rectifier K$_{V}$ channels

We have shown that RA inhibits voltage-gated CaV$_{2}$ channels, but how it affects ion channels to induce spike broadening and complex spiking is not yet known. As K$_{V}$ channels play an essential role in repolarization of the action potential, we next determined whether RA might induce spike broadening by modulating K$_{V}$ function. K$_{V}$ channels include the rapidly inactivating A-type channels and nonrapidly inactivating delayed rectifier channels, known to regulate spike broadening and burst firing in hippocampal neurons, respectively (2, 7). In Lymnaea neurons, A-type channels are blocked by 5 mM 4-aminopyridine (4-AP), whereas delayed rectifier channels are blocked by 50 mM tetroethyl ammonium (TEA) (30). In order to isolate A-type channel current (I$_{A}$), total potassium current was first recorded prior to perfusion of 4-AP. Following perfusion of 4-AP, the remaining 4-AP-insensitive current was subtracted from total IK$_{D}$, yielding I$_{A}$. Similarly, to isolate the delayed rectifier current (I$_{K_{D}}$) following TEA perfusion, the TEA-insensitive current was subtracted from total IK$_{D}$ to yield I$_{K_{D}}$. To determine whether RA affected I$_{A}$ or I$_{K_{D}}$, VF neurons were cultured overnight in the presence or the absence of RA, and the IV relationships for both I$_{A}$ and I$_{K_{D}}$ were established.

Raw recordings of I$_{A}$ and I$_{K_{D}}$ following exposure to RA (or DMSO) are shown in Figure 7, A and B, respectively. A two-way ANOVA of either I$_{A}$ or I$_{K_{D}}$ revealed a significant effect of treatment for I$_{A}$ ($F_{(1,420)} = 6.059; p = 0.014$) and I$_{K_{D}}$ ($F_{(1,665)} = 9.264; p = 0.002$). However, post hoc analysis revealed nonsignificant effects at all potentials for I$_{A}$, suggesting RA had only minimal effects on I$_{A}$ (Fig. 7A). The IV relationship in Figure 7B indicates that RA significantly reduced I$_{K_{D}}$ at potentials from +25 to +55 mV, compared with controls (DMSO). As Ca$^{2+}$-activated and Na$^{+}$-activated K$^{+}$ channels might also play a role in action potential repolarization and be affected by RA, we also examined these currents. However, isolation of either Ca$^{2+}$-activated K channels (RA: 1.809 ± 3.0 pA/pF; DMSO: 3.417 ± 4.6 pA/pF) or Na$^{+}$-activated K$^{+}$ channels (RA: 4.227 ± 2.3 pA/pF; DMSO: 2.504 ± 4.0 pA/pF) yielded extremely small peak current densities and were thus not studied further.
Figure 6. RA limits Ca$^{2+}$ influx through Ca$\text{v}$$\text{2}$, but not Ca$\text{v}$$\text{1}$, channels during modulated spiking. A, raw recordings of Ca$\text{v}$$\text{2}$ I$_{\text{Ca}}$ (top traces) during control and modulated voltage protocols in the presence of 0.02% DMSO (black, left) or 2 μM RA (red, right). B, graph indicating that the modulated voltage protocol significantly enhanced Ca$\text{v}$$\text{2}$ I$_{\text{Ca}}$ compared with the control protocol but did so to a greater extent in the presence of DMSO (green, n = 11) than in the presence of RA (red, n = 10). * represents the comparison between modulated protocols in RA (red circles) and DMSO (green triangles) (*p ≤ 0.05; **p ≤ 0.01). ! represents the enhanced I$_{\text{Ca}}$ between the modulated protocol in RA (red circles) and the control protocol in DMSO (black squares). !p ≤ 0.05; !!p ≤ 0.01; !!!p ≤ 0.001. C, the modulated voltage protocol (in RA and DMSO) significantly enhanced Ca$\text{v}$$\text{1}$ I$_{\text{Ca}}$ compared with the control voltage protocol. However, there was no significant effect of RA on I$_{\text{Ca}}$ compared with DMSO, regardless of voltage protocol. # represents a comparison between modulated and control protocols in both (B) and (C) (#p ≤ 0.05; ##p ≤ 0.01; and ###p ≤ 0.001). Ca$\text{v}$$\text{x}$, voltage-gated Ca$^{2+}$ channel; DMSO, dimethyl sulfoxide; I$_{\text{Ca}}$, calcium current; RA, retinoic acid.
Overall, these data suggest that RA inhibits delayed rectifier channels (I_{KD}), and that this effect might contribute to the RA-induced spike broadening and activity-dependent complex spiking.

**RA enhances K\textsubscript{V} channel inactivation**

Raw recordings previously shown in Figure 7, A and B (arrows) suggest that RA might enhance the channel inactivation of I\textsubscript{A} and I\textsubscript{KD}. K\textsubscript{V} channel inactivation would limit the number of channels available for action potential repolarization and if modulated by RA, might mediate the activity-dependent changes in firing. K\textsubscript{V} channels can undergo several types of inactivation; A-type channels undergo a rapid form of inactivation, which is coupled to channel opening (referred to as N-type inactivation), as well as a slower form of inactivation, referred to as U-type inactivation (31–33). Of these two forms of inactivation, delayed rectifier channels might only undergo U-type inactivation, if any (31–33). This U-type inactivation occurs at intermediate voltages but is reduced at more positive voltages, generating a typical “U” shape in inactivation curves (31–33).

VF neurons were again cultured overnight in the presence or the absence of RA, and the voltage dependence of channel inactivation of both I\textsubscript{A} and I\textsubscript{KD} was examined. Following an initial “control” pulse to +35 mV, cells were stepped from a holding potential of −115 mV to voltages ranging from −115 to +55 mV (for 500 ms) to induce inactivation, followed by a 200 ms “test” pulse to +35 mV (Fig. 8A). I\textsubscript{A} displayed an inactivation curve characteristic of N-type inactivation (Fig. 8B), whereas I\textsubscript{KD} displayed an inactivation curve typical of U-type inactivation (Fig. 8C).

A two-way ANOVA of I\textsubscript{A} inactivation revealed a significant interaction between treatment and voltage (F\textsubscript{(34,420)} = 2.444; p < 0.001). Figure 8B shows that RA enhanced the inactivation of I\textsubscript{A} at potentials between −5 and +55 mV, though had no effect on the voltage of half-maximal inactivation (RA: −12.27 ± 7.8 mV; DMSO: −13.393 ± 10.5 mV; p = 0.825) or the slope factor (RA: −14.99 ± 2.3; DMSO: −11.38 ± 2.6; p = 0.053).
Similarly, a two-way ANOVA of I_{KD} inactivation also revealed a significant interaction between treatment and voltage ($F_{(34,420)} = 3.689; p < 0.001$). RA enhanced inactivation of the delayed rectifier channel ($I_{KD}$) at potentials between +5 and +55 mV (Fig. 8C) but had no effect on the voltage of half-maximal inactivation (RA: $-3.037 \pm 9.9$ mV; DMSO: $-6.753 \pm 9.1$ mV; $t = 0.867; p = 0.397$) but did significantly increase the slope factor (RA: $-5.542 \pm 1.3$; DMSO: $-4.258 \pm 1.1; t = 2.434; p = 0.026$).

Together, these data suggest that RA enhanced channel inactivation of both A-type ($I_A$) and delayed rectifiers ($I_{KD}$) but did not alter the voltage dependence of inactivation for either channel type.

**RA enhances activity-dependent closed state channel inactivation**

Recovery from inactivation is also an important property that determines the availability of K_v channels for repolarization during neuronal activity. Importantly, K_v channels can exist in a closed state (occurring at hyperpolarized potentials and characterized by all four voltage sensors being in the inactive position) or an intermediate-closed state (occurring at intermediate voltages and characterized by at least one of four voltage sensors being in the active position). N-type inactivation subsequently allows for recovery from both closed and intermediate-closed states. U-type inactivation preferentially occurs from intermediate-closed states, and recovery only occurs at potentials that promote the fully closed state (32, 33). This property of U-type inactivation generates a situation whereby repeated opening and closing of the channels results in inactivation, which has been proposed to occur during ongoing neuronal firing (32).

We determined whether RA influences recovery from inactivation at either an intermediate voltage or a hyperpolarized voltage. The protocol used to determine recovery from inactivation is shown in Figure 9A. After a control pulse to +35 mV, cells were stepped from a holding potential of $-115$ mV to $+35$ mV for 500 ms to induce inactivation and were subsequently stepped down to a potential of either $-45$ mV (intermediate voltage) or $-115$ mV (hyperpolarized voltage) for a variable duration between 0 and 200 ms in 10 ms intervals, followed again by a test pulse to +35 mV.

Recovery from inactivation was normalized to the baseline level of inactivation (at $t = 0$ ms) at both $-45$ mV and $-115$ mV. A two-way ANOVA for recovery from inactivation of A-type channels ($I_A$) revealed a significant effect of treatment at both $-45$ mV ($F_{(1,252)} = 19.54; p < 0.001$) and $-115$ mV ($F_{(1,252)} = 34.688; p < 0.001$). However, the recovery curve at $-45$ mV was not significantly affected by RA at any particular time point, indicating that RA had minimal effects on recovery from inactivation of $I_A$ (Fig. 9B). Post hoc analysis of the normalized recovery from inactivation of A-type channels ($I_A$) at $-115$ mV revealed that RA enhanced the recovery from inactivation, compared with DMSO (Fig. 9C).

The same analysis was next performed for $I_{KD}$. The recovery from inactivation was again normalized to the baseline level of inactivation (at $t = 0$ ms). Interestingly, at the recovery potential of $-45$ mV, the current became further reduced over time (Fig. 9D), and there was no apparent recovery from inactivation (following treatment with either RA or DMSO). These data strongly suggest that the delayed rectifier channels exhibited intermediate closed-state inactivation at $-45$ mV. A two-way ANOVA of the normalized recovery from inactivation for $I_{KD}$ revealed a significant effect of RA treatment at $-45$ mV ($F_{(1,336)} = 74.261; p < 0.001$). Specifically, RA significantly reduced $I_{KD}$ at $-45$ mV (between 100 and 200 ms), suggesting that RA enhanced the intermediate closed-state inactivation at $-45$ mV (Fig. 9D).
In contrast to −45 mV, recovery from inactivation was apparent at the recovery pulse to −115 mV (Fig. 9E), indicating voltage dependence in recovery from inactivation. A two-way ANOVA of the normalized recovery from inactivation for IKD revealed a significant interaction between RA treatment and recovery time at −115 mV (F(20,378) = 2.622; p < 0.001). At −115 mV, RA significantly enhanced recovery from inactivation of IKD at later time points (130–200 ms) (Fig. 9E).

In summary, the data obtained at −45 mV (but not −115 mV) suggest that RA enhanced U-type inactivation of IKD, coupled to both channel opening and the intermediate-closed state, an effect that is activity dependent. This would likely generate an effect whereby repeated channel open and closure during firing activity in the presence of RA could lead to progressively enhanced inactivation of delayed rectifier channels (IKD), potentially leading to activity-dependent complex spiking.

**RA inhibits delayed rectifier channels during neuronal activity**

If, as we suggest previously, the RA-enhanced inactivation of IKV leads to spike broadening and activity-dependent complex spiking, then we predict that exposure to RA would lead to a reduction of IKD during ongoing neuronal activity. To test this hypothesis, we again utilized our control and “modulated” voltage protocols to record either IA or IKD in VF neurons exposed overnight to RA (or DMSO). We first examined whether exposure to RA affected IK during either the modulated (Fig. 10, A and B) or the control (Fig. 10C) voltage protocols. Statistical tests revealed that though the modulated voltage protocol enhanced IK at −45 mV compared with the control protocol (*p ≤ 0.05; **p ≤ 0.01; and ***p ≤ 0.001, compared with DMSO. DMSO, dimethyl sulfoxide; RA, retinoic acid.

In contrast, we found that exposure to RA significantly reduced IK at spikes 3 to 9 of the modulated voltage protocol (Fig. 10, D and E, * symbols). Exposure to RA also reduced IK at spikes 7 and 8 of the control voltage protocol (Fig. 10F, * symbols). These data suggest that exposure to RA inhibited the delayed rectifier channel activity during ongoing neural activity, possibly because of enhanced inactivation as a result of repeated opening and closing of the
Figure 10. RA inhibits delayed rectifier channels during neuronal firing. A, raw representative recordings of A-type channel currents (I_A; top traces) during modulated voltage protocols (bottom traces) in the presence of either 0.02% DMSO (black, left) or 2 μM RA (red, right). B and C, although the modulated protocols exhibited increased current area densities compared with the control protocols, RA had no significant effect on I_A compared with DMSO, using either the modulated (B) or the control (C) voltage protocol. D, raw recordings of delayed rectifier channel currents (I_Kr) during the modulated voltage protocol in the presence of either 0.02% DMSO (black, left) or 2 μM RA (red, right). E and F, RA significantly reduced delayed rectifier current area densities during both the modulated voltage protocol (E) and the control voltage protocol (F). G, the average current in RA, subtracted from the average current in DMSO, plotted for both control and modulated voltage, increased as a function of spike number, suggesting that the RA-induced inhibition of the delayed rectifier channel occurs during ongoing neuronal activity and increases with spike number. * represents the comparison between modulated protocols in RA (red circles) and DMSO (green triangles). **p < 0.01; ***p ≤ 0.001. # represents a comparison between modulated and control protocols; #p ≤ 0.05; ##p ≤ 0.01; and ###p ≤ 0.001. DMSO, dimethyl sulfoxide; RA, retinoic acid.
Ion channel neuromodulation by retinoic acid

channel. If this were indeed the case, then the RA-mediated reduction in \( I_{KD} \) should be more pronounced with increased neuronal activity and thus, with increasing spike number during the voltage protocols (both control and modulated protocols). Hence, the difference between \( I_{KD} \) generated in DMSO and RA for each protocol should also increase with each spike. To examine this, the average \( I_{KD} \) in the presence of RA was subtracted from the average \( I_{KD} \) in DMSO and plotted for both the modulated and control voltage protocols (Fig. 10G). Indeed, the difference in the averaged \( I_{KD} \) exhibited an upward trajectory as a function of spike number for both the control protocol (gray line) and the modulated spike protocol (blue line). These data thus suggest that the RA-induced inhibition of the delayed rectifier channel (but not the A-type channel) occurs during ongoing neuronal activity and increases with spike number.

Overall, we conclude that RA primarily produces activity-dependent modulation of neuronal firing by enhancing U-type inactivation of delayed rectifier channels, which likely occurs as a result of repeated opening and closing of the channel during such ongoing neuronal activity.

Discussion

In this study, we provide evidence for two neuromodulatory effects of RA that might ultimately affect synaptic transmission. We first show that RA induces complex spiking in an activity-dependent manner (an effect that occurs in multiple cell types) and provide evidence that the underlying mechanism involves enhanced inactivation of delayed rectifier \( K_v \) channels. However, RA concurrently inhibits \( Ca_{V2} \) channels, and we also determined how this interacts with the effects of modulated firing. Our data provide novel evidence to suggest that RA signaling modulates both neuronal firing activity and \( Ca_{V2} \) channel gating to tune Ca\(^{2+}\) signaling mediated by \( Ca_{V2} \) channels.

RA alters the firing properties of neurons by acting on \( K_v \) channels

The average concentration of all-trans RA in the \textit{Lymnaea} CNS was previously estimated at \(~0.693 \, \mu\text{M}\) (26). It is therefore conceivable that local concentrations (in neuronal microdomains) might be comparable to those used here to modulate firing properties and channel activity. We determined that RA concentrations as low as 1 \( \mu\text{M} \) induced spike broadening and activity-dependent complex spiking. The acute RA-mediated spike broadening and complex firing in \textit{Lymnaea} neurons was previously shown to be both transcriptionally (23) and translationally independent (34) and did not appear to involve PKA or phospholipase C activation (34). We now provide novel evidence for RA-induced inhibition of neuronal delayed rectifier (\( K_v(D) \)) channels, which we propose is largely responsible for spike broadening and activity-dependent complex spiking (though the precise molecular signaling pathway by which RA inhibits these channels remains to be determined). To our knowledge, an RA-mediated reduction in delayed rectifier K-channel currents has not previously been shown in the nervous system (in either vertebrates or invertebrates), though has been demonstrated in human lymphocytes (35).

We propose that the RA-induced reduction in delayed rectifier activity might be responsible for the RA-induced spike broadening, as both were found to be independent of activity. However, RA also enhanced inactivation (of both delayed rectifier and A-type channels), and as inactivation is an activity-dependent process, this provides evidence for activity-dependent regulation of both \( K_v \) channels by RA. Though we cannot rule out a role for the A-type channel inactivation, we propose that RA likely induces the activity-dependent complex spiking via enhanced inactivation of the delayed rectifier (\( K_v(D) \)) channels. In particular, we show that RA enhanced U-type inactivation, a form of inactivation that occurs at intermediate voltages (corresponding to open and intermediate-closed states). Previous research has shown that delayed rectifier channels expressed in non-neuronal cells (human embryonic kidney cells) exhibit U-type inactivation, and the authors proposed that this may contribute to firing properties during ongoing neuronal activity (32). Repeated opening and closure of the channel during ongoing neuronal activity would result in progressive inactivation because of repeatedly transitioning through “intermediate states.” To our knowledge, this is the first evidence for U-type inactivation of delayed rectifier channels in neurons, and we propose that this U-type inactivation underlies the activity-dependent complex spiking induced by RA.

Activity-dependent complex spiking may be a widespread effect as it occurred in many different cell types (peptidergic and dopaminergic), involved in different behaviors. However, there may be some cell-type specificity, as we show that complex spiking was not activity dependent in the RPA respiratory motor neurons. This differential sensitivity might result from differential expression of either retinoid receptors or ion channels sensitive to RA (and/or the presence or the absence of compensatory mechanisms). The complex spiking was also not activity dependent at higher concentrations, possibly because of greater effects of these higher concentrations on \( K_v \) channels (35).

Delayed rectifier channels are highly conserved throughout evolution, and both invertebrate- and vertebrate-delayed rectifier channels undergo U-type inactivation when expressed in insect and non-neuronal vertebrate cell lines, respectively (31, 32, 36), suggesting that RA perturbation of these channels and U-type inactivation might modulate neuronal firing activity in both invertebrate and vertebrate neurons. Indeed, inhibiting delayed rectifier channels (such as \( K_v(2) \)) in rat hippocampal neurons enhances burst firing properties. Interestingly, such burst firing occurs \textit{in vivo} during spatial navigation and learning and memory (7, 24, 37), suggesting a possible mechanism for the known enhancing effects of retinoids on learning and memory in vertebrates (and invertebrates).

RA signaling regulates activity-dependent neuronal signaling

Both complex spiking and activity-dependent synaptic plasticity are proposed to be the basis for learning and
Ion channel neuromodulation by retinoic acid

memory. In vertebrates, RA signaling is important for activity-dependent forms of synaptic plasticity, such as LTP and LTD. Indeed, perturbations of retinoid receptors have been shown to affect both synaptic plasticity and behavior; in rodents, downregulation of retinoid receptors (such as retinoic acid receptor β and/or retinoid X receptor γ) disrupts either LTP or LTD and results in impaired novel object recognition and/or impaired working and spatial memories (11, 13, 14, 38).

RA signaling also affects other forms of plasticity in the vertebrate hippocampus, such as homeostatic plasticity, a form of metaplasticity that maintains neuronal activity within an optimal range. Specifically, in response to reduced synaptic activity, RA increases the synthesis (and insertion) of CaV2-permeable α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors, an effect that is dependent on NMDA receptor activity (8, 15, 16). Interestingly, this RA-mediated increase in α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor expression then blocks subsequent LTP (16), suggesting that the effects of RA on different forms of plasticity are complex, and may have multiple interacting and/or opposing effects. To date, no studies have examined whether RA affects CaV or Kv channel currents in vertebrate neurons and/or synapses.

We have previously shown that RA signaling is also required for normal long-term associative memory formation in Lymnaea (21). It is thus feasible that the RA-mediated changes in neuronal firing and CaV2 channel activity might contribute to synaptic plasticity in this molluscan species. Indeed, we have previously shown that inhibiting RA signaling (with retinoid receptor antagonists) produced voltage-dependent inhibition of Lymnaea CaV2 channels (29), an ubiquitous form of presynaptic plasticity in vertebrate neurons. The RA-mediated spike broadening shown here is also reminiscent of the spike broadening in presynaptic neurons occurring following behavioral training, and which mediates synaptic facilitation in the mollusc Aplysia (39). An important question to consider next is how changes in neural activity (such as during learning) might lead to RA-mediated spike broadening. We propose that RA synthesis might be regulated by neuronal activity. Indeed, RA synthesis is affected by activity at hippocampal synapses (17, 27), and in the Lymnaea CNS, we have evidence that expression of the enzyme responsible for RA synthesis increases during memory formation (40).

Multiple forms of RA-mediated neuromodulation coexist

Multiple forms of RA-mediated modulation might coexist in the same neuron to produce similar outcomes on synaptic plasticity. For example, during homeostatic plasticity in vertebrates, RA signaling enhances excitatory synaptic transmission onto mouse hippocampal neurons whilst independently, but simultaneously, reducing inhibitory synaptic transmission onto the same cells by enhancing endocytosis of the postsynaptic gamma-aminobutyric acid receptors. The coexistence of these differing effects of RA leads to a shift in synaptic weight to produce an overall increase in excitability (18).

Our study has now also provided evidence for multiple modes of RA-mediated modulation coexisting and interacting in invertebrate neurons, though in this case, the effects oppose each other. We show that a concentration of RA (2 μM) that produces activity-dependent complex spike broadening (enhancing CaV2 influx) also inhibits CaV2 influx through CaV2 channels. We further provide evidence that these differing effects of RA interact. By using playback of modulated spiking protocols in the presence or the absence of RA, we found that RA-mediated inhibition of CaV2 channels limits the enhanced CaV2 influx that occurs during RA-mediated spike broadening and complex spiking. However, when the CaV2 channel currents were examined in isolation, the inhibition of CaV2 by RA prevented the increase in CaV2 influx during spike broadening. Indeed, it was only during complex spiking that the enhanced influx of CaV2 dominated and overcame CaV2 channel inhibition.

Despite these multimodal modulatory effects of RA, it is unlikely that the RA-mediated inhibition of CaV2 channels is directly responsible for the RA-mediated changes in neuronal firing. CaV channels were blocked whilst examining actions of RA on I_KD, and thus changes in CaV2 influx (through Cav2s) could not account for these results. Furthermore, reduced CaV2 influx is unlikely to affect CaV2-activated K channels, as these particular channels generate very little current in these cells. RA-mediated effects on spiking might, however, have led to a homeostatic reduction in CaV2 activity (or vice versa), though we consider this unlikely as both effects of RA (on spiking and CaV channels) can occur rapidly and over similar timescales (34). We thus propose that although RA-mediated modulation of spike activity and inhibition of CaV2 channels coexist in the same cell type, that they are likely independent effects.

Differential effects of RA on CaV1s versus CaV2s

We determined that spike broadening and complex spiking enhanced CaV2 influx through CaV2 channels, though simultaneous RA exposure did not limit this, because RA did not inhibit CaV2 influx via CaV1. Interestingly, we previously found that higher concentrations of RA (5 μM) did inhibit CaV1 channels, though using barium instead of physiological levels of CaV2 (25). This may have affected the outcome, if we consider the possibility that the presence of CaV2 might influence the actions of RA on CaV1 channels.

Because of the different locations and roles of CaV1 and CaV2 channels in neuronal microdomains, our results suggest a scenario whereby RA-induced modulation of spiking might enhance CaV2 signaling involved in gene regulation (CaV1s) but would limit the enhancement in CaV2-mediated synaptic signaling. Moreover, CaV1 channels have been implicated in presynaptic plasticity mechanisms, such as presynaptic LTP (41), as well as neuropeptide release (42), suggesting that specific regulation of CaV2 (but not CaV1) channels by RA during activity-dependent changes in spiking may ultimately target selective CaV2 signaling pathways or functions. RA-mediated modulation of neuronal activity and its differential modulation of CaV1 and CaV2 channels may thus be a mechanism to fine-tune information processing in different compartments in invertebrate or vertebrate neurons.
**Ion channel neuromodulation by retinoic acid**

**Possible consequences for synaptic transmission and plasticity**

In both vertebrate and invertebrate neurons, depolarization during action potential firing activates CaV2 channels present in presynaptic terminals, with the resulting Ca\(^{2+}\) influx mediating neurotransmitter release. When the duration of depolarization is prolonged, during either spike broadening or complex spiking, it would likely increase the fidelity and magnitude of synaptic transmission. Indeed, we found that RA-mediated complex spiking enhanced CaV2-mediated I\(_{\text{Ca}}\), though RA-mediated inhibition of CaV2 channels compensated for any increase in I\(_{\text{Ca}}\) during spike broadening. As such, the system might act as a high-pass filter, whereby only activity leading to complex spiking enhances synaptic output. The ability of RA to limit enhancement of I\(_{\text{Ca}}\) (via inhibition of CaV2\(\alpha\)s) might also prevent synaptic “runaway.” Indeed, it has been shown that RA signaling can limit LTP in mice following environmental enrichment (43), and if RA signaling is disrupted, mice exhibit synaptic runaway, resulting in reduced cognitive flexibility. It should also be noted that during playback of the normal control-spiking protocols, RA also induced a reduction in CaV2 currents, which, albeit small and nonsignificant, might also have physiological consequences on synaptic transmission. It is thus possible that RA might also inhibit CaV2-mediated synaptic transmission during “normal” spike trains.

In this study, we present the first cellular evidence for activity-dependent modulation by RA in an invertebrate CNS. However, whether the RA-mediated activity-dependent complex spiking plays a direct role in synaptic plasticity and/or memory formation requires further evaluation. In mouse hippocampal neurons, complex spiking, such as burst firing of presynaptic neurons, is involved in memory formation (24). In contrast, burst firing in postsynaptic neurons can induce homeostatic synaptic depression (44), and postsynaptic spike broadening can lead to LTD (45). Thus, determining the exact role of RA-mediated spike broadening and activity-dependent burst firing during synaptic transmission, by examining RAs effects in both presynaptic and postsynaptic cells, will be needed.

It is very likely that both short-term and long-term consequences of complex spiking on synaptic transmission will be synapse specific and might depend on the release properties and dynamics of that particular synapse. Furthermore, the increases in Ca\(^{2+}\) influx during complex spiking will likely modulate various kinase pathways and/or local protein synthesis, as well as potentially mediate neuropeptide or neurotrophin release, in addition to the aforementioned gene transcription. To add a further level of complexity, RA is known to enhance neurotransmitter release from *Xenopus* motor neurons, in a manner that is independent of extracellular calcium (46). Thus, determining the precise role of RA-induced activity-dependent complex spiking in synaptic modulation and plasticity will require a thorough study of a number of different synapses, utilizing different neurotransmitters.

In summary, the ability of RA to inhibit CaV2 channels must coexist with its ability to induce spike broadening and activity-dependent complex spiking, which ultimately enhances Ca\(^{2+}\) influx. This concurrent inhibition of CaV2 channels thus influences and fine-tunes Ca\(^{2+}\) signaling during ongoing neuronal activity.

**Experimental procedures**

**Animals**

The gastropod mollusc, *L. stagnalis*, utilized in these experiments was bred at Brock University and maintained at room temperature on a fixed 12:12 h light–dark cycle in aerated dechlorinated water. Animals were fed a combination of romaine lettuce and Nutrafin Max Spirulina fish food (Hagen) daily. CNS from adult animals (20–23 mm in shell length) were used for cell culture.

**Cell culture**

All dissections were performed under sterile conditions as previously described (25, 29). Briefly, *Lymnaea* were anesthetized through incubation in saline containing 25% Listerine (containing menthol, 0.042% w/v). *Lymnaea* were subsequently pinned in a dissection dish containing antibiotic saline (normal saline containing 225 μg/ml gentamicin [Sigma–Aldrich]) and dissected to expose the CNS consisting of the central ring ganglia, which was removed and given three 10 min washes in antibiotic saline. The CNS was treated with trypsin (Sigma–Aldrich; 2 mg/ml) in defined medium (DM; 50% Leibovitz’s L-15 medium; Gibco) for 18 to 19 min at 21 °C and subsequently treated with trypsin inhibitor (Sigma–Aldrich; 2 mg/ml in DM) for 10 min at 21 °C. The CNSs were then pinned out in high-osmolality DM, followed by removal of the outer sheath of connective tissue. The inner sheath was subsequently removed to expose the neurons. Individually identified neurons were removed from the ganglia using a fire-polished glass pipette coated with Sigmaclone (Sigma–Aldrich) to prevent cell adhesion. Gentle suction was applied via a microsyringe (Gilmont) to remove individual neuronal cell bodies from the CNS. Individual neurons were subsequently plated in DM on plastic culture dishes coated with poly-L-lysine (Sigma–Aldrich). Cells were then treated with all-trans RA (Sigma–Aldrich), prepared as a 10\(^{-2}\) M stock solution in 100% DMSO, and diluted in DM to produce a final bath concentration ranging from 1 to 5 μM. Control cells were treated with an equivalent concentration of DMSO. The different cell types used in this study included VF peptidergic neurons, the dopaminergic RPeD1, RPB, and RPA cells. VF neurons were the predominant cell type used. Between five and eight VF cells were generally isolated from one CNS and plated in either one or two culture dishes. Most experiments used only one cell/dish (unless stated otherwise), and so no more than 1 to 2 cells from each CNS were utilized for recordings.

The firing properties of cultured neurons were assessed using current-clamp electrophysiology, whereas the biophysical properties of Ca\(_V\) and K\(_V\) channels were assessed using whole-cell voltage clamp electrophysiology (see later for details). All recordings from cultured cells were performed at...
Ion channel neuromodulation by retinoic acid

Current clamp

Current-clamp recordings were performed in DM. Patch pipettes with a resistance between 2 and 6 MΩ were filled with an internal solution containing 2 mM Mg-ATP, 0.1 mM GTP–Tris, 5 mM EGTA, 1 mM CaCl₂, 10 mM Hepes, 60 mM potassium gluconate with a pH of 7.4 achieved using potassium hydroxide (30). To stimulate action potential firing, cells were held at 0 pA for 4 s followed by current steps between −100 and +400 pA in 25 pA intervals for 5 s. Current-clamp recordings were not limited to one cell per dish, but only cells that had an RMP more negative than −40 mV were used. Recordings were performed on cells with an access resistance of <15 MΩ and performed at room temperature. Because of the variability in capacitance between VF neurons (100–300 pF) and between the four different cell types used in this study (100–600 pF), the firing properties of cells were assessed at the first current step in which at least three action potentials were elicited (rheobase 1) as well as the three subsequent current steps (referred to as rheobases 2–4). To assess spike broadening, the half-width of the first three spikes in a particular current step was averaged, as done previously (23).

To confirm there was an increase in the number of spikes (events) across rheobases 1 to 4, the number of spikes was counted for each rheobase (independent of whether it was a complex spike) and statistically analyzed to confirm an increase in activity across rheobases. To then assess activity-dependent complex spiking, the number of complex spikes was counted at rheobases 1 to 4. A cell was deemed to exhibit complex spiking if a doublet, triplet, burst, or plateau potential occurred at any of the four rheobases.

Additional electrophysiological parameters measured included the voltage at which the peak of the action potential occurred, the peak voltage of the AHP, RMP, and input resistance. The equation, $R = \frac{\Delta V}{\Delta I}$, was used to calculate the input resistance (obtained from hyperpolarizing current pulses) in each cell.

Caᵥ channel recordings

ICₐ for both voltage-clamp and action potential clamp was assessed using an external solution containing 4.1 mM CaCl₂, 40 mM tetrabutylammonium chloride, 1.5 mM MgCl₂, 10 mM Hepes, and 5 mM 4-AP, at a pH of 7.9, achieved using TEA-Cl. Patch electrodes with a resistance between 2 and 6 MΩ were filled with internal solution containing 29 mM CsCl, 1 mM CaCl₂, 2 mM MgATP, 0.1 mM GTP–Tris, 5 mM EGTA, 10 mM Hepes, and 60 mM potassium gluconate with a pH of 7.4 achieved using cesium hydroxide.

Recordings were performed on cells with an access resistance of <15 Ω with series resistance compensation to 85%. As spike firing characteristics could not be recorded in the exact same cell as ICₐ in order to ensure that the RA-mediated effects were occurring in parallel, twoVF cells from the same CNS were consistently plated together in one dish and exposed to identical treatments. One cell was first used to confirm the RA-mediated effects on spike broadening (current clamp), whereas the second cell was then used to record RA-mediated effects on ICₐ (voltage clamp).

Characterization of Caᵥ channel subtype using nifedipine

The selective L-type Ca²⁺ channel blocker, nifedipine, was used to determine the effects of RA on L-type versus non–L-type Caᵥ channels. Cells were incubated overnight in either 2 μM RA or 0.02% DMSO (controls). A stock solution of nifedipine was prepared at a concentration of 10⁻² M using 100% DMSO. Neurons that had been exposed to either RA or DMSO were then acutely perfused with external solution containing 0.1% DMSO (as a vehicle control), and an IV relationship was obtained in DMSO, which represented total ICₐ. This solution was then replaced with external solution containing 10 μM nifedipine.

VF neurons were held at −115 mV and stepped to +5 mV for 200 ms once every 30 s while the inhibitor, nifedipine, was applied to the bath, until peak inhibition was attained. The IV relationship of the remaining nifedipine-insensitive ICₐ through Caᵥ2 (non–L-type) was established. The nifedipine-sensitive ICₐ through Caᵥ1 (L type) was then determined by subtracting nifedipine-insensitive ICₐ from total ICₐ. Subtraction was performed offline using Clampfit 9.2 (Molecular Devices). The voltage dependence of channel activation and the voltage of half-maximal activation were determined as described later. To assess the amount of channel rundown that might occur during nifedipine-mediated L-type Ca²⁺ channel block, additional control experiments were performed in which cells were only exposed to 0.1% DMSO, and rundown was assessed over a similar period over which nifedipine block would normally occur. Voltage steps to +5 mV (from a holding potential of −115 mV) were conducted every 30 s over a time course of 10 min, and revealed no subsequent rundown of ICₐ (an actual 4.4% increase in ICₐ occurred; n = 6).

Kᵥ recordings

KV channel currents (IK) were recorded using the same internal solution as used for current-clamp recordings. Prior to obtaining recordings of K’ currents in voltage-clamp mode, voltage recordings of the firing properties of VF neurons (treated with either RA or DMSO) were obtained in current-clamp mode. This was done in the presence of DM using a modified version of the current-clamp protocol described previously, to ensure that RA consistently produced spike broadening and complex spiking. The DM was then replaced with the appropriate K’ channel external solution, depending
Ion channel neuromodulation by retinoic acid

on the K⁺ channel type being isolated. In this study, we isolated four different types of K⁺ channels: A-type KV channels, delayed rectifier KV channels, Na+-activated K⁺ channels, and Ca²⁺-activated K⁺ channels. To isolate Na+-activated K⁺ channels, K⁺ currents were first recorded in an external solution containing 40 mM NaCl, 1.7 mM KCl, 4.1 mM CaCl₂, 1.5 mM MgCl₂, 10 mM Hepes, and pH 7.9 with potassium hydroxide (Na⁺-containing external solution). This external solution was then replaced with Na+-free external solution containing 5 mM 4-AP, and IK was again recorded in Na+-free external solution from K⁺ currents obtained in Na+-containing external solution. Ca²⁺-activated K⁺ channel currents were obtained by subtracting K⁺ currents obtained in Na⁺-free external solution from K⁺ currents obtained in Na⁺-containing external solution. Ca²⁺-activated K⁺ channel currents were obtained by first recording IK in Na⁺-free external solution (Total IK). This Na⁺-free external solution was then replaced with Na⁺-free external solution containing 30 μM cadmium to block ICa, and IK was subsequently established. Subtraction of IK remaining in the presence of cadmium from total IK yielded Ca²⁺-activated K⁺ currents.

Isolation of A-type IK was achieved using 4-AP. IK was first recorded in Na⁺-free external solution (total IK). This Na⁺-free external solution was then replaced with Na⁺-free external solution containing 5 mM 4-AP, and IK was again established. Subtraction of IK remaining in the presence of 4-AP from total IK yielded A-type IK. Isolation of delayed rectifier (IKD) was achieved using TEA. Total IK was first recorded in Na⁺-free external solution containing 30 μM cadmium as calcium-activated K⁺ channels are also inhibited by TEA. This external solution was then replaced with Na⁺-free external solution containing 30 μM cadmium and 50 mM TEA, and IK was again established. Subtraction of IK remaining in the presence of TEA from total IK yielded delayed rectifier IKD.

IV relationship

To determine the IV relationship for ICa and IK, cells were held at −115 mV and stepped to +5 mV for ICa or +35 mV for IK for 200 ms to generate the control ICa or IK (Icontrol). Icontrol was measured with each sweep of the protocol to account for any rundown that may have occurred over the course of the recording. Voltage-dependent inactivation was achieved by holding cells at −115 mV and stepping to membrane potentials between −115 and +55 mV for 500 ms in 5 mV increments. The membrane potential was then immediately stepped to +5 mV for ICa or +35 mV for IK for 200 ms to generate the test current (Itest) (25). To generate a steady-state inactivation curve, Itest/Icontrol was calculated for each test potential. Values for each test potential were then fit in Clampfit using the Boltzmann equation. In the case of delayed rectifier channels, as a result of U-type inactivation, Boltzmann fits were only performed on voltages between −115 and +25 mV, which is where the inactivation peaks, but this fit line was extended to +55 mV in Figure 8C to illustrate the U-type inactivation. The voltage of half-maximal inactivation and the slope factor produced during each treatment condition were compared using an unpaired t test or a Mann–Whitney rank sum test, as appropriate.

Voltage dependence of channel inactivation

To determine the voltage dependence of CaV and Kv channel inactivation, cells were held at −115 mV and stepped to +5 mV for ICa or +35 mV for IK for 200 ms to generate the control ICa or IK (Icontrol). Icontrol was calculated using linear extrapolation of the IV curve at potentials more depolarized than that at which the peak current occurs. Conductance values for each voltage were normalized to the maximal conductance, gmax, for each neuron. The values of g/gmax were fit to the Boltzmann equation:

\[ f(V) = \frac{g_{\text{max}}}{1 + e^{\frac{V_{\text{mid}} - V}{V_c}}} + C \]

where Vmid represents the voltage of half-maximal activation, V represents the membrane potential, Vc represents the slope factor, and C represents a constant. The voltage of half-maximal activation and slope factor produced for each treatment condition were compared using an unpaired t test or a Mann–Whitney rank sum test, as appropriate.

Recovery from channel inactivation

To determine the recovery from inactivation, cells were held at −115 mV and stepped to +5 mV for ICa or +35 mV for IK for 200 ms to generate Icontrol. Voltage-dependent inactivation was achieved by depolarizing the membrane potential to +5 mV for ICa or +35 mV for IK for 500 ms. The membrane potential was then returned to −115 mV for ICa (or to both −115 mV and −45 mV for IK) for a variable duration of time ranging from 0 to 1 s in 50 ms intervals for ICa or 0 to 200 ms in 10 ms intervals for IK, to allow the channels to recover from inactivation. The membrane potential was then stepped to +5 mV for
I_{Ca} or +35 mV for I_K for 200 ms to generate the test current (I_{test}). I_{test} produced during each recovery period was then normalized to I_{control} and plotted against the duration of time allowed for recovery. The resultant recovery from inactivation curve was compared across treatment conditions using a two-way ANOVA. To determine the time constant, the recovery from inactivation curve was fit with the exponential equation. The time constant of the recovery from inactivation was compared between treatment groups using an unpaired t test or a Mann–Whitney rank sum test.

**Action potential clamp**

To determine whether the RA-mediated spike broadening and complex spiking influenced CaV and K channels, we utilized whole-cell action potential clamp. Representative current-clamp voltage traces at rheobase 4 of a cell pretreated with either 2 μM RA (“modulated” protocol) or 0.02% DMSO (“control” protocol) were used as the voltage stimulus. VF neurons were exposed to either 2 μM RA or 0.02% DMSO overnight, whole-cell voltage clamped, and exposed to both the “control” protocol and the “modulated” voltage protocols. Both the control and modulated protocols consisted of nine spikes. Cells were held at −115 mV for 5 s to remove any inactivation and then stepped to −55 mV for 5 s to allow ion channels to occupy the appropriate gating states at the approximate RMP. Cells were then stimulated with the control and modulated voltage protocols. To remove the noise in the current recording that was generated as a result of the noise in the voltage recordings (used as the voltage protocols), a 200 Hz low-pass filter was applied to each recording offline in Clampfit. Each recording was manually inspected before and after this filter was applied to ensure only noise was removed. The current area that was coincident with each spike in both protocols was quantified in Clampfit and normalized to the capacitance of each cell, and the current remaining at the end of the action potential firing protocol was subtracted as leak current.

Four experimental conditions were analyzed: (1) overnight exposure to 2 μM RA, followed by the control protocol; (2) overnight exposure to 2 μM RA, followed by the modulated voltage protocol; (3) overnight exposure to 0.02% DMSO, followed by the control protocol; and (4) overnight exposure to 0.02% DMSO, followed by the modulated voltage protocol. Groups were compared across all nine spikes (of the protocol) using a two-way ANOVA. To determine the contribution of capacitative current (during total I_{Ca} and CaV2 I_{Ca}) during action potential clamp, control experiments were performed in Ca^{2+} channel external solution containing 0 mM Ca^{2+} and 30 μM cadmium to block all Ca^{2+} influx and revealed that the capacitative current was negligible.

**Statistical analysis**

All statistical analyses were performed using SigmaStat 3.2 (Sysstat), and graphs were generated using GraphPad Prism 5.03 (GraphPad Software, Inc). Values are presented as mean ± SD, and differences were deemed significant when p ≤ 0.05.

**Data availability**

All data are contained within the article.

**Author contributions**—E. d. H. and G. E. S. conceptualization; E. d. H. methodology; E. d. H. validation; E. d. H. formal analysis; E. d. H. and G. E. S. investigation; G. E. S. resources; E. d. H. and G. E. S. writing—original draft; E. d. H. and G. E. S. visualization; G. E. S. supervision; G. E. S. funding acquisition.

**Funding and additional information**—This work was supported by a Discovery Grant from The Natural Sciences and Engineering Research Council of Canada (02825; to G. E. S.). E. d. H. was supported by an Ontario Graduate Scholarship.

**Conflict of interest**—The authors declare that they have no conflicts of interest with the contents of this article.

**Abbreviations**—The abbreviations used are: 4-AP, 4-aminoptyridine; AHP, afterhyperpolarization; CaV, voltage-gated Ca^{2+} channel; CNS, central nervous system; DM, defined medium; DMSO, dimethyl sulfoxide; I_{A}, A-type channel current; I_{Kd}, delayed rectifier current; IV, current–voltage; K_V, voltage-gated K^{+} channel; LTD, long-term depression; LTP, long-term potentiation; RA, retinoic acid; RMP, resting membrane potential; RPA, right parietal A; RPB, right parietal B; RPd1, right pedal dorsal 1; TEA, tetraethyl ammonium; VF, visceral F.

**References**

1. Nanou, E., and Catterall, W. A. (2018) Calcium channels, synaptic plasticity, and neuropsychiatric disease. *Neuron* 98, 466–481
2. Cho, I. H., Panzera, L. C., Chin, M., Alpizar, S. A., Olveda, G. E., Hill, R. A., and Hoppa, M. B. (2020) The potassium channel subunit K_{β1} serves as a major control point for synaptic facilitation. *Proc. Natl. Acad. Sci. U. S. A.* 117, 29937–29947
3. Hell, J. W., Westenbroek, R. E., Warner, C., Ahlijianan, M. K., Prystay, W., Gilbert, M. M., Snutch, T. P., and Catterall, W. A. (1993) Identification and differential subcellular localization of the neuronal class C and class D L-type calcium channel a1 subunits. *J. Cell Biol.* 123, 949–962
4. Wheeler, D. G., Groth, R. D., Ma, H., Barrett, C. F., Owen, S. F., Safa, P., and Tsien, R. W. (2012) Ca_{V1} and Ca_{V2} channels engage distinct modes of Ca^{2+} signaling to control CREB-dependent gene expression. *Cell 149*, 1112–1124
5. Cheong, E., and Shin, H. S. (2013) T-type Ca^{2+} channels in normal and abnormal brain functions. *Physiol. Rev.* 93, 961–992
6. Gutman, G. A., Chandy, K. G., Grissmer, S., Lazdunski, M., McKinnon, D., Pardo, L. A., Robertson, G. A., Rudy, B., Sanguinetti, M. C., Stuhmer, W., and Wang, X. (2005) International union of pharmacology. LIIL. Nomenclature and molecular relationships of voltage-gated potassium channels. *Pharmacol. Rev.* 57, 473–508
7. Raus Balind, S., Mago, A., Ahmadi, M., Kis, N., Varga-Nemeth, Z., Lorincz, A., and Makara, J. K. (2019) Diverse synaptic and dendritic mechanisms of complex spike burst generation in hippocampal CA3 pyramidal neurons. *Nat. Commun.* 10, 1859
8. Chen, L., Lau, A. G., and Sarti, F. (2014) Synaptic retinoic acid signaling and homeostatic synaptic plasticity. *Neuropsychopharmacology* 78, 3–12
9. Cunningham, T. J., and Duester, G. (2015) Mechanisms of retinoic acid signaling and its roles in organ and limb development. *Nat. Rev. Mol. Cell Biol.* 16, 110–123
Ion channel neuromodulation by retinoic acid

Ochoa, W. F., Torrecillas, A., Fita, I., Verdaguer, N., Corbalan-Garcia, S., and Gomez-Fernandez, I. C. (2003) Retinoic acid binds to the C2-domain of protein kinase Ca. *Biochemistry* **42**, 8774–8779

Chiang, M.-Y., Misner, D., Kempermann, G., Schikorski, T., Giguere, V., Suev, H. M., Gage, F. H., Stevens, C. F., and Evans, R. M. (1998) An essential role for retinoid receptors RARβ and RXRα in long-term potentiation and depression. *Neuron* **21**, 1353–1361

Cocco, S., Díaz, G., Stancampiano, R., Diana, A., Carta, M., Curreli, R., Sarais, L., and Fadda, F. (2002) Vitamin A deficiency produces spatial learning and memory impairment in rats. *Neuroscience* **115**, 475–482

Nomoto, M., Takeda, Y., Uchida, S., Mitsuda, K., Enomoto, H., Saito, K., Choi, T., Watabe, A. M., Kobayashi, S., Masushige, S., Manabe, T., and Kida, S. (2012) Dysfunction of the RAR/RXR signaling pathway in the forebrain impairs hippocampal memory and synaptic plasticity. *Mol. Brain* **5**, 8

Upreti, C., Woodruff, C. M., Zhang, X.-L., Yin, M. J., Zhou, Z.-Y., Pagano, A. M., Behanian, D. S., Yin, D., Kandel, E. R., Stanton, P. K., and Nicholls, R. G. (2015) Loss of retinoid X receptor gamma subunit impairs group 1 mGluR-mediated electrophysiological responses and group 1 mGluR-dependent behaviours. *Sci. Rep.* **11**, 5552

Aoto, J., Nam, C. L., Poon, M. M., Ting, P., and Chen, L. (2008) Synaptic signaling by all-trans retinoic acid in homeostatic synaptic plasticity. *Neuron* **60**, 308–320

Arendt, K. L., Zhang, Y., Junrod, S., Malenka, R. C., Sudhof, T. C., and Chen, L. (2015) Retinoic acid and LTP recruit postsynaptic AMPA receptors using distinct SNARE-dependent mechanisms. *Neuron* **86**, 442–456

Arendt, K. L., Zhang, Z., Ganesan, S., Hintze, M., Shin, M. M., Tang, Y., Cho, A., Graef, I. A., and Chen, L. (2015) Calcineurin mediates homeostatic synaptic plasticity by regulating retinoic acid synthesis. *Proc. Natl. Acad. Sci. U. S. A.* **112**, E5744–E5752

Sarti, F., Zhang, Z., Schroeder, J., and Chen, L. (2013) Rapid suppression of inhibitory transmission by retinoic acid. *J. Neurosci.* **33**, 11440–11450

Yee, A. X., and Chen, L. (2016) Differential regulation of spontaneous and evoked inhibitory synaptic transmission in somatosensory cortex by retinoic acid receptor RARα. *J. Neurosci.* **36**, 445–452

Zhong, L. R., Chen, X., Park, E., Sudhof, T. C., and Chen, L. (2018) Retinoic acid receptor RARα-dependent synaptic signaling mediates homeostatic synaptic plasticity at the inhibitory synapses of mouse visual cortex. *J. Neurosci.* **38**, 10454–10466

Rothwell, C. M., and Spencer, G. E. (2014) Retinoid signaling is necessary for, and promotes long-term memory formation following operant conditioning. *Neurobiol. Learn. Mem.* **114**, 127–140

Rothwell, C. M., de Hoog, E., and Spencer, G. E. (2017) The role of retinoid acid in the formation and modulation of invertebrate central synapses. *J. Neurophysiol.* **117**, 692–704

Vesprini, N. D., and Spencer, G. E. (2014) Retinoic acid induces changes in retinoid receptor-based signaling plays a role in voltage-dependent inhibition of invertebrate voltage-gated Ca2+ channels. *J. Biol. Chem.* **294**, 10076–10093

Sakakibara, M., Okuda, F., Nomura, K., Watanabe, K., Meng, H., Hori-koshi, T., and Lukowiak, K. (2005) Potassium currents in isolated stato-cyst neurons and RPeD1 in the pond snail, Lymnaea stagnalis. *J. Neurophysiol.* **94**, 3884–3892

Klemic, K. G., Kirsch, G. E., and Jones, S. W. (2001) U-type inactivation of Kv3.1 and Shaker potassium channels. *Biophys. J.* **81**, 814–826

Klemic, K. G., Shieh, C.-C., Kirsch, G. E., and Jones, S. W. (1998) Inactivation of Kv2.1 potassium channels. *Biophys. J.* **74**, 1779–1789

Bett, G. C. L., Dinga-Madou, I., Zhou, Q., Bondarenko, V. E., and Ras-musson, R. L. (2011) A model of the interaction between N-type and C-type inactivation in Kv1.4 channels. *Biophys. J.* **100**, 11–21

Vesprini, N. D., Dawson, T. F., Yuan, Y., Bruce, D., and Spencer, G. E. (2015) Retinoic acid affects calcium signaling in adult molluscan neurons. *J. Neurophysiol.* **113**, 172–181

Sidell, N., and Schlachter, L. (1986) Retinoic acid blocks potassium channels in human lymphocytes. *Biochem. Biophys. Res. Commun.* **138**, 560–567

Carrillo, E., Arias-Olguin, I. I., Islas, L. D., and Gomez-Lagunas, F. (2013) Shab K+ channel slow inactivation: A test for U-type inactivation and a hypothesis regarding K+-facilitated inactivation mechanisms. *Channels* **7**, 97–108

Epstein, J., Brecht, M., and Lee, A. K. (2011) Intracellular determinants of hippocampal CA1 place and silent cell activity in a novel environment. *Neuron* **70**, 109–120

Wiertzycz, M., Meziane, H., Sutter, A., Ghyselinck, N., Chapman, P. F., Chambon, P., and Krezé, W. (2005) Working memory deficits in retinoid X receptor γ-deficient mice. *Learn. Mem.* **12**, 318–326

Byrne, J. H., and Kandel, E. R. (1996) Presynaptic facilitation revisited: State and time dependence. *J. Neurosci.* **16**, 425–435

Rothwell, C. M., Simmons, J., Peters, G., and Spencer, G. E. (2014) Novel interactive effects of darkness and retinoic signaling in the ability to form long-term memory following aversive operant conditioning. *Neurobiol. Learn. Mem.* **114**, 251–263

Fourcaudot, E., Gambino, F., Cassassus, G., Poulain, B., Humeau, Y., and Luthi, A. (2009) L-type voltage-dependent Ca2+ channels mediate expression of presynaptic LTP in amygdala. *Nat. Neuroscience* **12**, 1093–1095

Hsu, I.-U., Linsley, J. W., Zhang, X., Varineau, J. E., Berkhoudt, D. A., Reid, L. E., Lum, M. C., Orzel, A. M., Leflein, A., Xu, H., Collins, C. A., Hume, R. I., levitan, E. S., and Kuwada, J. Y. (2020) Stac protein regulates release of neuropeptides. *Proc. Natl. Acad. Sci. U. S. A.* **117**, 29914–29924

Hsu, Y.-T., Li, J., Wu, D., Sudhof, T. C., and Chen, L. (2019) Synthetic retinoic acid signaling mediates mTOR-dependent multiplicity that controls hippocampal learning. *Proc. Natl. Acad. Sci. U. S. A.* **116**, 7113–7122

Chang, C. W., Willkerson, J. R., Hale, C. F., Gibson, J. R., and Huber, K. M. (2017) Distinct stages of synapse elimination are induced by burst firing of CA1 neurons and differentially require MEF2A/D. *Elife* **6**, e26278

Zhou, Y.-D., Acker, C.-D., Nestoff, T. I., Sen, K., and White, J. A. (2005) Increasing Ca2+ transients by broadening postsynaptic action potentials enhances timing-dependent synaptic depression. *Proc. Natl. Acad. Sci. U. S. A.* **102**, 19121–19125

Liu, J.-C., Ho, S.-Y., Chen, M.-R., Liao, Y.-P., Chiu, W.-T., and Kang, K.-H. (2005) A rapid nongenomic pathway facilitates the synaptic transmission induced by retinoic acid at the developing synapse. *J. Cell Sci.* **118**, 4721–4730