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Developmental Biology 294 (2006) 39–49
DEVELOPMENTAL BIOLOGY
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Retinoic acid induces neurite outgrowth and growth cone turning in invertebrate neurons

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Received for publication 24 August 2005; revised 22 November 2005; accepted 14 February 2006

Abstract

Identification of molecules involved in neurite outgrowth during development and/or regeneration is a major goal in the field of neuroscience. Retinoic acid (RA) is a biologically important metabolite of vitamin A that acts as a trophic factor and has been implicated in neurite outgrowth and regeneration in many vertebrate species. Although abundant in the CNS of many vertebrates, the precise role of RA in neural regeneration has yet to be determined. Moreover, very little information is available regarding the role of RA in invertebrate nervous systems. Here, we demonstrate for the first time that RA induces neurite outgrowth from invertebrate neurons. Using individually identified neurons isolated from the CNS of *Lymnaea stagnalis*, we demonstrated that a significantly greater proportion of cells produced neurite outgrowth in RA. RA also extended the duration of time that cells remained electrically excitable in vitro, and we showed that exogenously applied RA acted as a chemoattractive factor and induced growth cone turning toward the source of RA. This is the first demonstration that RA can induce turning of an individual growth cone. These data strongly suggest that the actions of RA on neurite outgrowth and cell survival are highly conserved across species.

Keywords: Regeneration; Electrophysiology; *Lymnaea stagnalis*; Invertebrate; Retinoids; Electrical excitability; Trophic factors; Chemotaxis; Neurite outgrowth; Cell culture

Introduction

Retinoic acid (RA) is a naturally occurring, active metabolite of vitamin A that has recently been described as a trophic factor capable of inducing neurite outgrowth (Hunter et al., 1991; Maden et al., 1996; Corcoran et al., 2002). It is a small non-proteinaceous, lipophilic molecule that diffuses into cells and acts on ligand-activated transcription factors to influence gene activity. These transcription factors include the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs), and the target genes include cell surface molecules, neurotrophins, cytokines, as well as the retinoid receptors themselves (reviewed in Gudas et al., 1994; Mey and McCaffery, 2004). Alteration in gene expression in turn contributes to neuronal outgrowth during development and regeneration. For example, RA increases axonal outgrowth from embryonic spinal cord (Wuarin et al., 1990; Maden et al., 1998), dorsal root ganglia (DRG) (Corcoran et al., 2000), cerebellum (Yamamoto et al., 1996) and sympathetic ganglia (Plum et al., 2001) in vitro. RA also promotes axonal regeneration from differentiated spinal cord neurons in adult newts (Prince and Carlone, 2003; Dmetrichuk et al., 2005) and in synergy with some neurotrophins, induces outgrowth from differentiated retinal ganglion cells (Mey and Rombach, 1999). Many growth factors that promote neurite extension are also thought to guide developing and/or regenerating neurites by inducing growth cone turning (Song et al., 1997; McFarlane and Holt, 1997). In addition to inducing neurite outgrowth, RA has recently been implicated as a chemotropic molecule, guiding neurite outgrowth from dissociated cultures of embryonic chick neural tube (Maden et al., 1998), as well as the regenerating adult newt spinal cord in vitro (Dmetrichuk et al., 2005).

It has previously been shown that many factors that function in a trophic or chemotropic manner in vertebrates do so also in invertebrates (Tessier-Lavigne and Goodman, 1996). Specifically, it is now well established that various axon guidance molecules such as semaphorins, netrins, slits and ephrins, as well as their respective receptors, are phylogenetically well conserved

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0012-1606/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2006.02.018

and exhibit similar functional properties in vertebrates and invertebrates (Goodman, 1994; Garbe and Bashaw, 2004). RA has been shown to exert morphogenic effects during development in invertebrates (Créton et al., 1993), suggesting that the actions of RA are conserved across species. However, whether RA can induce neurite outgrowth or exert chemotropic effects in invertebrate neurons has not yet been studied.

Here, we aim to take advantage of a molluscan neuronal culture system to determine the role of RA in neurite outgrowth and growth cone turning during regeneration. The CNS of the pond snail *Lymnaea stagnalis* has previously proven a useful model system for the study of regeneration, neurite outgrowth and growth cone guidance (Syed et al., 1990, 1992; Spencer et al., 1996, 2000; Wildering et al., 2001). Neurons in the adult brain can regenerate in vivo (Syed et al., 1992) as well as in vitro (Syed et al., 1990). When single identified neurons are isolated from the adult CNS and plated in cell culture and provided with the appropriate trophic support, they can regenerate their neuritic processes. In this study, we isolated single identified Visceral F (VF) neurons from the adult central nervous system (CNS) and investigated whether RA induces neurite outgrowth and/or growth cone turning from these cells in vitro. We have shown for the first time that RA is capable of inducing neurite outgrowth from cultured invertebrate neurons as well as extending the duration of time that the cells remain electrically excitable in vitro. We have also shown for the first time in any species that RA can attract and induce turning of a single identified growth cone in vitro.

Materials and methods

Cell culture

L. stagnalis were laboratory bred, kept in aerated, artificial pond water and fed lettuce. All cell culture procedures were performed as described previously (Syed et al., 1990; Spencer et al., 2000). Briefly, the central ring ganglia were isolated under sterile conditions, and after a number of antibiotic washes and subsequent enzymatic treatment, pinned down in a dissection dish and bathed in high osmolarity (L-15 derived) defined medium (DM) (Ridgway et al., 1991). The connective tissue sheath surrounding the ganglia was removed using a pair of fine forceps, and identified Visceral F (VF) somata were individually extracted by applying gentle suction via a fire-polished pipette. The individually identifiable neurons were then plated directly on poly-L-lysine-coated dishes containing DM or (where stated) brain-conditioned medium (CM) (Wong et al., 1981). In DM alone, *Lymnaea* neurons do not normally extend neurites (Spencer et al., 1996), but regenerate their processes if cultured in CM (Syed et al., 1990; Spencer et al., 2000). CM contains many (as yet unidentified) trophic factors.

All-*trans* RA (Sigma, Toronto, Canada) was dissolved in absolute ethanol (EtOH) and added to the culture dishes to give a final bath concentration of 10^{-7} M RA. This concentration has previously been shown effective in inducing neurite outgrowth in vertebrate preparations (Maden et al., 1998; Corcoran and Maden, 1999; Dmetrichuk et al., 2005). Control dishes contained equivalent concentrations of EtOH in DM (0.001% EtOH) or DM alone. RA or EtOH was added by an experimenter other than that who isolated the neurons. All dishes were placed in the dark immediately following addition of RA or EtOH and were maintained at 22°C.

Analysis of neurite outgrowth

In order to analyze the effects of RA on the induction of neurite outgrowth, cells were plated in DM only (in the absence of brain-conditioned medium and thus the absence of any other trophic factors). Neurite outgrowth from cells incubated in RA, control vehicle or DM alone was scored 2, 3 and 4 days after plating. Cells were viewed with an inverted microscope (Zeiss Axiovert 200), and time-lapse imaging/software (Northern Eclipse) was used to monitor and analyze the extent of neuronal outgrowth. In order to be included in the analysis, neurons must have adhered to the culture substrate (in the absence of adhesion, no outgrowth would occur). The number and length of primary neurites emerging from each neuron were monitored at each time point. A 2-way repeated measures analysis of variance (2-way RM ANOVA) and Dunn's (Bonferroni corrected) post hoc test were used to determine statistical significance in the number and length of neurites in RA versus control conditions. Chi-squared tests were used to determine significance in the number of cells that produced outgrowth in each condition.

Electrophysiology

For intracellular recordings, conventional electrophysiological techniques were used as described previously (Syed et al., 1990; Spencer et al., 2000). Glass microelectrodes (resistance 20–40 MΩ) containing a saturated solution of K_2SO_4 were used to impale the neurons. The electrophysiological signals were amplified (IR-283, Cygnus Technology) and displayed using a data acquisition system designed by T. MacDonald (Brock University Electronics Department). Recordings were made from cultured neurons plated in RA, EtOH or DM. For some analyses, different cells were impaled on days 2, 3 and 4 of culture. A 2-way ANOVA and Bonferroni post hoc analysis were performed to compare resting membrane potentials of the cells between groups over time. Results were expressed as mean ± SEM, and differences were deemed significant if $P < 0.05$. Chi-squared tests were performed to compare the number of cells that fired action potentials following incubation in RA versus EtOH control. In a separate set of experiments, the same cells were impaled on day 2 and day 6 of culture. However, only cells that fired on day 2 (spontaneous, or in response to current injection) were analyzed again on day 6. The person performing the electrophysiological recordings was unaware of the cell culture conditions (RA versus controls). Cells were assessed for their ability to fire spontaneous or induced action potentials (0.1 to 0.5 pA depolarizing current injection). Chi-squared tests were performed to compare the number of cells that fired action potentials following incubation in RA versus EtOH control. Results were expressed as percent cells fired, and differences were deemed significant if $P < 0.05$.

Growth cone turning assays

For growth cone turning assays, cells were cultured either in DM or CM with RA (10^{-7} M final bath concentration). Growth cones were monitored for at least 1 h prior to exposure to a local gradient of RA. A pipette containing RA (10^{-5} M; Sigma, Toronto, Canada) was placed in close proximity (approximately 150 μm) to the growth cone, and RA was pressure ejected using an Eppendorf–Femtojet (10 min continuous pulses at 6–15 hPa, with 5 min between, for 1.5–3 h; pipette tip diameter: 3–7 μm). For control experiments, an equivalent concentration of the vehicle solution (0.1% EtOH in DM), or DM alone, was focally applied in the same manner as RA. In some experiments, the direction of the source of RA was switched from one side of the growth cone to the other following initial turning of the growth cone (within 1–2 h). Images were captured, and growth cone turning was analyzed using Northern Eclipse Software. Data were analyzed using a one-way ANOVA and Bonferroni's post hoc test.

Results

In order to determine whether RA could induce neurite outgrowth from isolated molluscan neurons, individually identified Visceral F (VF) neurons were isolated from the *Lymnaea* CNS and plated in DM in the presence or absence of RA (10^{-7} M). This concentration was previously used to examine RA's effects on eye formation in *Lymnaea* (Créton et al., 1993) and has proved effective in inducing neurite outgrowth from vertebrate preparations (Maden et al., 1998; Corcoran and Maden, 1999;

Dmetrichuk et al., 2005). Neurite outgrowth was monitored and assessed 2, 3 and 4 days after initial plating, and both the number and length of primary neurites extending from all cells (whether grown or not) were initially analyzed.

RA induced neurite outgrowth in a time-dependent manner

A 2-way RM ANOVA of the number of primary neurites from RA-treated, vehicle-treated cells and DM only controls revealed a significant interaction effect ($F(312,4) = 6.02$; $P = 0.0001$). Post hoc analysis revealed that the mean number of primary neurites was significantly greater in cells treated with RA ($n = 49$) than EtOH-treated controls ($n = 54$) or DM only controls ($n = 56$) at every time point (2, 3 and 4 days; $P < 0.01$; Fig. 1A). Furthermore, the RA-treated cells showed a time-dependent increase in the number of neurites per cell from day 2 to day 4 ($P < 0.01$). In addition to the number of primary neurites, the length of neurites was analyzed and a 2-way RM ANOVA showed a significant interaction effect ($F(312,4) = 29.46$; $P < 0.0001$). Post hoc analysis demonstrated that RA also induced significantly longer neurites than EtOH and DM controls at every time point (2, 3 and 4 days; $P < 0.01$; Fig. 1B). A time-dependent increase in the length of neurites from day 2 to day 4 was also observed in RA-treated cells only ($P < 0.01$). There were no significant differences ($P > 0.05$) between either the number or length of neurites in the EtOH control condition compared to the DM control condition at any time point (Figs. 1A and B).

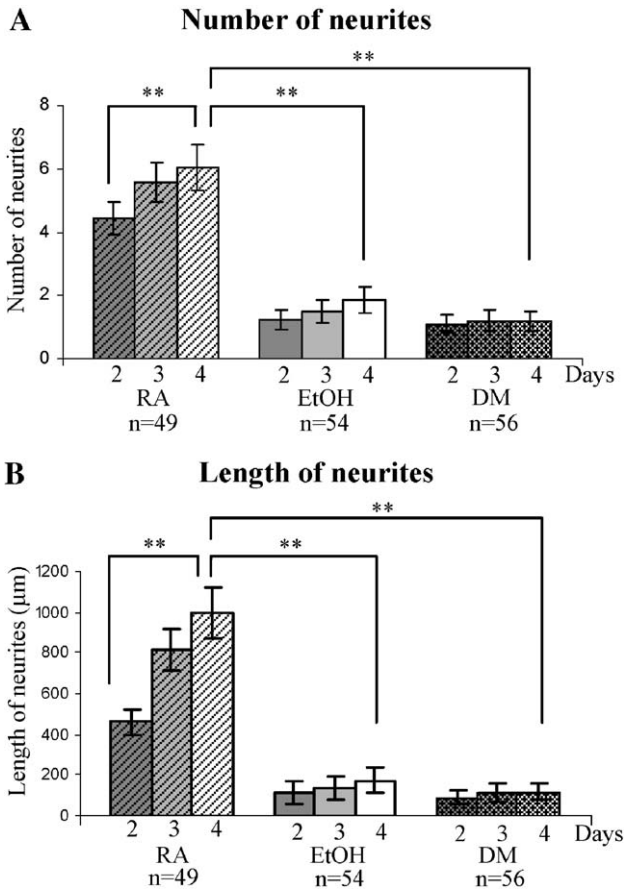

Fig. 1. Retinoic acid significantly increased neurite outgrowth. All cells plated in either RA or control (EtOH, DM only) conditions were assessed for neurite outgrowth (whether grown or not). Cells cultured in 10^{-7} M RA ($n = 49$) showed significantly more outgrowth than cells cultured in vehicle alone ($n = 54$) or DM only ($n = 56$). (A) The number of primary neurites/cell was significantly greater on days 2, 3 and 4 in RA compared to controls. (B) The length of primary neurites/cell was also significantly greater on days 2, 3 and 4 in RA compared to controls. $^{**}P < 0.01$. There were no significant differences ($P > 0.05$) in either the number or length of neurites between EtOH and DM at any time points.

The above data clearly indicate that, when all cultured cells were analyzed (regardless of whether they produced outgrowth or not), neurite outgrowth from cultured *Lymnaea* neurons was significantly greater in RA compared to vehicle or DM controls. However, we next needed to determine whether RA was *inducing* or *enhancing* the outgrowth from cells.

In order to determine whether RA was inducing more outgrowth from cells, we counted the number of individual cells that exhibited outgrowth after either 2, 3 or 4 days in culture. We found that significantly more VF cells exhibited neurite outgrowth in RA at each time point than in EtOH or DM control conditions ($P < 0.0001$). Representative examples of cells cultured in each condition are shown in Fig. 2A. The values for the number of cells that grew on day 2 are shown in Fig. 2B (RA: 89.8%, $n = 44$ of 49; EtOH: 29.6%, $n = 16$ of 54; DM: 30.4%, $n = 17$ of 56; Chi-square: $P < 0.0001$). There was no significant difference between number of cells grown when cultured in EtOH versus DM alone at any time point (Chi-square: $P > 0.05$).

For this next part of the study, we wanted to determine whether RA *enhanced* outgrowth over time. We thus analyzed the growth *only* from cells that had produced outgrowth in RA or in either of the control conditions and analyzed the number and length of neurites at each time point. First of all, the 2-way RM ANOVA and post hoc analyses again showed that there were no significant differences between the length of neurites between control cells in EtOH versus those in DM at any time point ($P > 0.05$). However, when length of neurites was analyzed between RA and controls (Fig. 3A), a significant interaction effect was observed [$F(166,4) = 7.12$; $P < 0.0001$]. Fig. 3A shows that the length of neurites was significantly greater in RA ($n = 45$) versus EtOH ($n = 20$) and DM controls ($n = 21$) on days 3 and 4 ($P < 0.01$). There was also a time-dependent increase in length of neurites from day 2 to day 4 in RA only ($P < 0.01$). Furthermore, there was a significant difference in the number of neurites between RA and EtOH or DM controls on Day 4 ($P < 0.05$), as well as a significant time-dependent increase in the number of neurites in RA ($P < 0.01$; Fig. 3B). Fig. 4 shows a representative example of outgrowth from an RA-treated cell on day 2 (Ai), 3 (Aii) and 4 (Aiii), as well as representative examples of cells in EtOH (Fig. 4B) and DM only (Fig. 4C) that produced some neurite outgrowth on days 2, 3 and 4.

These data thus show that RA both induced outgrowth and enhanced the outgrowth from cultured neurons. Firstly, we showed that more cells regenerated neuritic processes in RA than in either control condition. Once cells had produced neurite outgrowth, RA significantly enhanced the length and number of these neurites over time. Due to this latter result, we

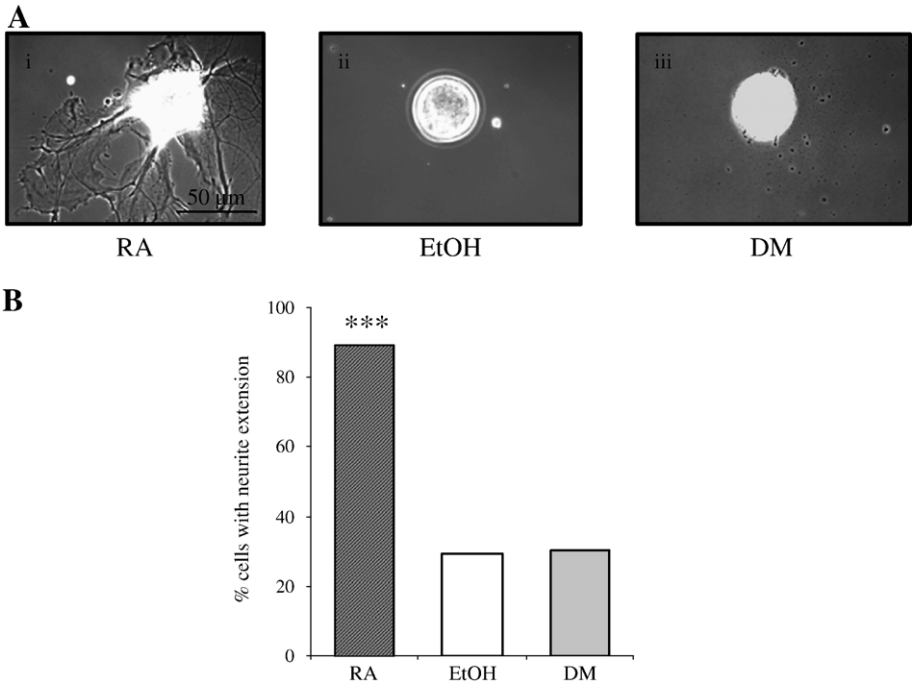

Fig. 2. More cells produced outgrowth in RA. (A) Representative examples of a cell showing outgrowth in RA (i), but not in EtOH (ii) or DM alone (iii). (B) Graph showing that the percentage of cells producing neurite outgrowth in RA on day 2 (10^{-7} M) was significantly greater than the percentage of cells producing outgrowth in vehicle control (EtOH) or DM alone. In RA, 44 of 49 cells produced outgrowth, in EtOH, 16 of 54 produced outgrowth, whereas in DM 17 of 56 cells produced outgrowth by day 2. ***$P < 0.0001$.

hypothesized that RA may be maintaining the viability of these neurons for longer periods in culture. To test this hypothesis, we next used electrophysiological techniques to determine whether cells treated in RA were more viable at each time point, compared to controls.

Cells retain their electrical excitability in RA over time

For this part of the study, a separate group of VF neurons was isolated and plated in DM, either in the presence or absence of RA (10^{-7} M). Standard intracellular recording techniques were used to determine whether RA prolonged the "viability" of these neurons in cell culture. Viability was assessed only from the measure of resting membrane potential (RMP), as well as the ability to retain electrical excitability (the ability to fire action potentials) over time. Initially, in the first series of experiments, different VF neurons were recorded at each time point (in order to avoid potential damage incurred from previous impalements of the cells at earlier time points). Different cells were thus used for recordings at 2, 3 and 4 days in vitro.

A two-way ANOVA and post hoc analysis of RMP values showed both a significant difference between RA and vehicle control groups (between-group effect: ($F(57,1) = 12.59$; $P < 0.001$) as well as a significant effect over time (within-group effect: ($F(57,2) = 6.425$; $P < 0.01$). That is, post hoc analysis showed that cells treated with RA had more negative RMPs than controls both on day 2 (RA $n = 11$, EtOH $n = 7$; $P < 0.05$) and day 4 (RA $n = 12$, EtOH $n = 9$; $P < 0.01$) (Fig. 5A). Control cells also showed a significant decline in RMP from day 3 ($n = 8$) to day 4 ($n = 9$; $P < 0.05$), which was not observed in the RA-treated cells ($P > 0.05$). When electrical excitability of these same VF cells was assessed, a Chi-squared test revealed that more cells fired action potentials (spontaneous or induced firing) after treatment with RA versus the EtOH controls, both on day 3 ($\chi^2 = 6.35$, $P < 0.05$) and day 4 of culture ($\chi^2 = 10.52$, $P < 0.01$; Fig. 5B). Of the 11 cells in RA that fired APs on day 4, 4 cells exhibited spontaneous firing compared to 2 cells in the vehicle control (EtOH). We also compared the EtOH controls to cells in DM only and found no significant difference between the number of cells firing (spontaneous or induced firing) in EtOH compared to DM ($n = 2$ of 9) on day 4 of culture ($\chi^2 = 0.00$, $P > 0.05$).

These data strongly suggest that, following incubation in RA, the VF neurons were less susceptible to physiological impairment and retained their electrical excitability over a longer period of time in vitro. However, one potential drawback of this experiment was that different cells were used for recordings on different days. We could therefore not be certain whether cells that failed to fire on day 4 had initially been "viable" on day 2 of culture.

In the next series of experiments (using a different group of VF cells), we thus aimed to determine whether cultured cells that were capable of firing on day 2 were also capable of firing for extended periods of time in RA. We also extended the time frame of recordings to determine the extent of the RA effect and recorded from neurons on day 2 and then again from the *same neurons* on day 6 of culture. VF cells were again plated in DM, in the presence or absence of RA. All cells were analyzed at day 2, but only cells that fired action potentials on day 2 (spontaneous or induced firing) were tested again on day 6. On day 2,

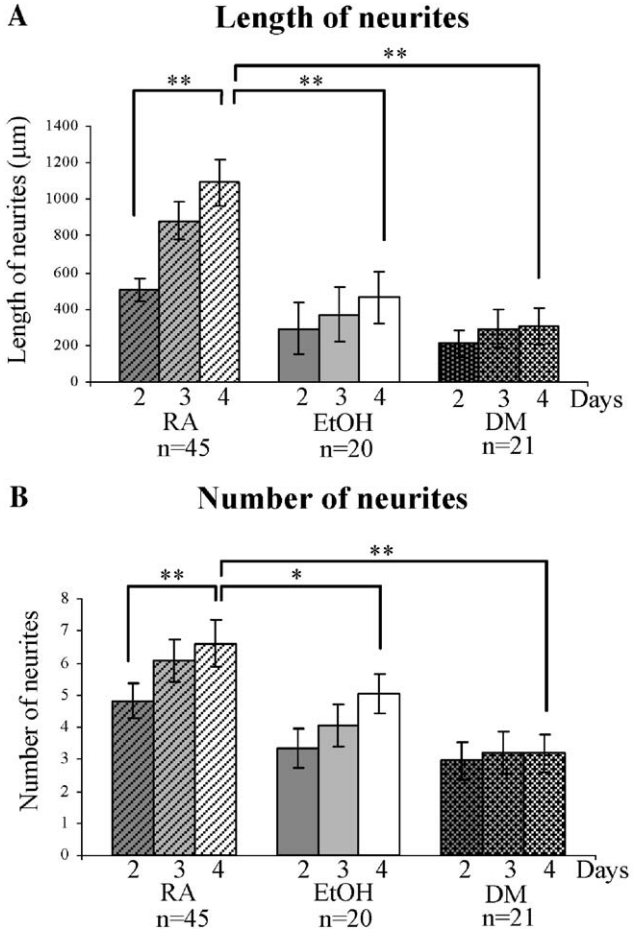

Fig. 3. RA increased the length and number of neurites compared to controls. When only cells that had grown in either condition were analyzed separately, it was found that the length (A) and the number (B) of neurites in RA (10^{-7} M) on day 4 were significantly greater than that of vehicle controls. RA also produced a time-dependent increase in both the length and number of neurites. *N* values represent cells grown by day 4. $^{**}P < 0.01$, $^{*}P < 0.05$.

we found that 66.7% of cells (10 of 15) spontaneously fired in RA, whereas the remaining 5 cells required current injection to induce firing. However, only 35.7% of cells (5 of 14) cultured in EtOH showed spontaneous firing on day 2; the remaining 9 cells required current injection to induce firing.

By day 6, we found that RA-treated cells were more likely to fire (spontaneous or induced) action potentials than controls (RA: $n = 10$ of 15; Controls: $n = 4$ of 14; $\chi^2 = 4.209$, $P < 0.05$). Fig. 6Ai illustrates a representative electrophysiological recording obtained from a VF cell cultured in 10^{-7} M RA after 2 days and 6 days in vitro. The cell fired (induced) action potentials at both times points following the injection of depolarizing current. In the absence of RA (Fig. 6Aii; vehicle control), a cell fired action potentials on day 2 but failed to fire action potentials in response to depolarizing current injection on day 6. Of the cells that fired on day 6, we also determined that significantly more RA-treated cells ($n = 8$ of 15) retained spontaneous electrical excitability (i.e., spontaneous firing) than controls ($n = 1$ of 14) ($\chi^2 = 9.6$, $P < 0.01$; Fig. 6B). When the outgrowth from these spontaneously active cells were analyzed, 62.5% (5 of 8) exhibited outgrowth in RA. The control cell (1 of 14) that fired spontaneously on day 6 in EtOH had not produced outgrowth. Furthermore, we found no significant difference between whether cells that had produced outgrowth fired action potentials on day 6 in either RA or EtOH ($\chi^2 = 0.60$; $P > 0.05$). Thus, it appears from these data that, though RA promoted the retention of spontaneous firing by day 6, this effect appeared to be independent of the presence of neurite outgrowth.

RA directed neurite outgrowth by inducing growth cone turning

The above data strongly suggest that RA exerted a trophic effect on VF cells by both inducing neurite outgrowth and extending the duration of electrical excitability of the cells in culture. There is evidence in the literature to suggest that many neurotrophic factors that promote survival and/or neurite outgrowth also act as chemotactic molecules during axon guidance (McFarlane and Holt, 1997). We next aimed to determine whether RA was capable of inducing growth cone turning of VF neurons in vitro.

VF neurons were cultured in RA and CM and following outgrowth (1 to 3 days) were tested for growth cone turning in response to an exogenous source of focally applied RA. Growth cones were first monitored for at least 1 h to ensure that no spontaneous changes in direction occurred. Exogenous RA (10^{-5} M) was then locally applied in a pulsatile manner to an individual VF growth cone, using a pipette positioned at an angle to the advancing growth cone (Fig. 7Ai). We found that RA was able to induce growth cone turning toward the source of RA ($n = 7$; Figs. 7Aiii, Aiv), whereas control applications of EtOH ($n = 12$) or DM only ($n = 10$) did not induce positive turning. As CM contains unidentified brain-derived trophic factors, we also repeated the above experiments in DM and showed that there were no apparent differences in the ability of RA to direct neurite outgrowth in either DM (mean = 64.3°, $n = 3$) or CM (mean = 66.9°, $n = 7$). When the DM and CM data were combined, we found that the mean angle of turning induced by RA was 66.1° ($n = 10$), whereas after control EtOH applications, it was −15.8° ($n = 12$) and −16.8° for DM applications ($n = 10$). A one-way ANOVA followed by post hoc analysis showed a significant difference in angle of turning between RA and both EtOH and DM controls ($P < 0.0001$), but no significant difference between EtOH and DM controls ($P > 0.05$). The individual turning angles for each growth cone in all conditions are graphed in Fig. 7B.

We next tested whether RA could re-direct the turning of the growth cone if the source of the RA was switched from one side of the growth cone to the other. Initial turning of the growth cone to RA (10^{-5} M) was produced within 1–3 h of RA application. Once an initial turning of the growth cone had been induced (mean angle of turning: 26°, $n = 3$ of 3), the RA was immediately applied from the opposite side of the growth cone. Following application of the RA from the opposite side of the growth cone, each growth cone tested subsequently changed its direction of outgrowth and again turned toward the source of RA (mean angle of turning toward RA: 48°, $n = 3$ of 3; Fig. 8). These experiments clearly showed that, if the source of RA was

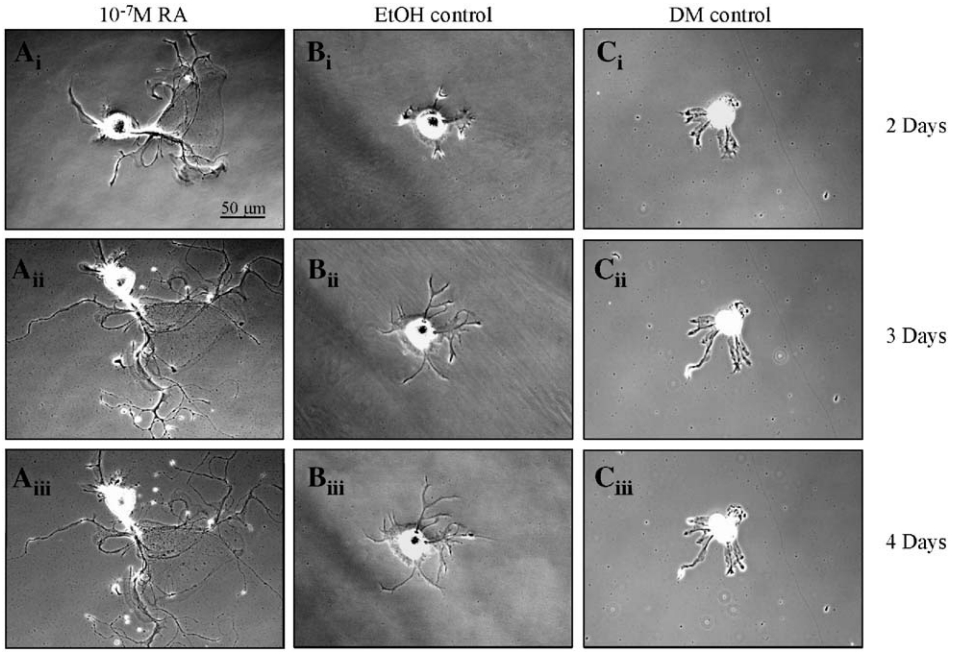

Fig. 4. Representative examples of neurite outgrowth from cultured neurons in all conditions. (A) Photomicrographs of a cell cultured in RA showing outgrowth after 2, 3 and 4 days in cell culture. Photomicrographs of cells that produced some outgrowth in control conditions are shown for comparison in B (EtOH) and C (DM only), on days 2, 3 and 4.

moved following initial turning, the growth cone would redirect its outgrowth and again move toward the source of RA.

Discussion

In this study, we demonstrated for the first time that RA can induce neurite outgrowth from invertebrate neurons. In the absence of any other trophic factors, RA induced significantly more cells to produce neurite outgrowth, and this outgrowth was significantly enhanced over time. That is, RA significantly enhanced both the number and length of neurites from cells in RA by day 4 in culture. Our results also support the notion that RA exerted trophic effects on the cells and maintained their electrical excitability and RMP in culture for a longer period of time. Thus, as is the case for many peptidergic trophic factors (such as the neurotrophins), RA appeared to enhance viability as well as induce outgrowth of neurons in vitro.

Previously, it has been difficult to assess survival versus growth-inducing effects of various trophic factors because most factors that promote cell survival also promote outgrowth. However, Goldberg et al. (2002) recently used retinal ganglion cells to elegantly show that axonal growth is not a default function of neuronal survival, but rather requires a specific signal. We propose that the same may be true for *Lymnaea* neurons and that RA did not induce outgrowth by merely enhancing survival of the neurons. For instance, there was no significant difference between the proportion of cells that fired on day 2 of culture in RA versus control conditions, yet a significantly greater proportion had sprouted in RA after 2 days. Though by no means conclusive, these data support the notion that RA exerted specific neurite-inducing effects, independent of its effects in prolonging viability of the neurons. These conclusions are further supported by the finding that a number of cells that retained spontaneous firing by day 6 in RA did not exhibit any neurite outgrowth. Though the mechanisms of these actions of RA have not yet been elucidated, it is possible that, like other trophic factors, RA uses different intracellular signaling pathways to mediate the different effects. RA has previously been shown to affect ion channel expression in various cells or cell lines, usually during differentiation. For example, in differentiating neuroblastoma cell lines, RA induced the appearance of an inward rectifying potassium channel (Tonini et al., 1999) as well as induced changes in sodium channel properties, affecting cell excitability (Toselli et al., 1996). It is quite possible that the effects of RA in prolonging membrane excitability in our cultured neurons may thus be a result of changes in various ion channel expressions. Furthermore, evidence suggests that inositol triphosphate receptors (which are important regulators of intracellular Ca^{2+}) are up-regulated following RA treatment (Bradford et al., 1992) and that RA can induce increases in intracellular [Ca^{2+}], likely via L- and/or N-type Ca^{2+} channels (Fukuhara et al., 1997; Gao et al., 1998). As changes in intracellular Ca^{2+} are important regulators of neurite outgrowth and growth cone behavior (Kater and Mills, 1991), the effects of RA on Ca^{2+} concentrations in *Lymnaea* neurons will need further investigation.

Though there are no previous reports of RA inducing outgrowth from invertebrate neurons, our results are consistent with findings from many vertebrate preparations. For example, Maden et al. (1998) found that RA significantly increased the proportion of cells extending neurites from embryonic chick neural tissue, and the concentrations of RA used in their studies

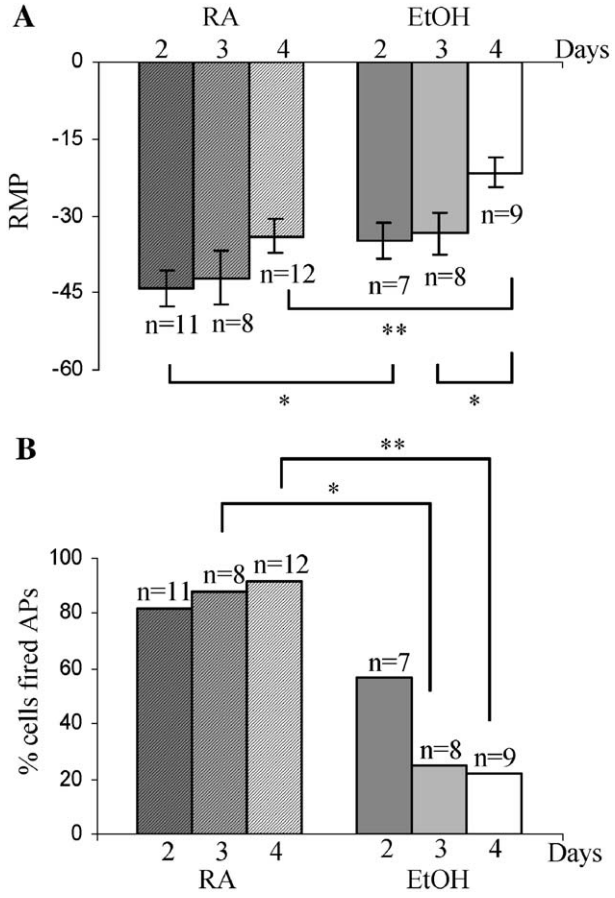

Fig. 5. RA extended the duration that cells were electrically excitable in culture. Standard intracellular recordings were made from cells incubated in RA or vehicle control on days 2, 3 and 4 of cell culture. Different cells were impaled on different days, and each cell was recorded from only once. (A) The resting membrane potential (RMP) of cells cultured in RA was maintained over time as only control cells showed a significant decline in RMP from day 3 to day 4. (B) The graph shows that the percentage of cells that were electrically excitable (spontaneous or induced firing) on days 3 and 4 of culture was significantly greater in RA than those cultured in vehicle control. *$P < 0.05$, **$P < 0.01$.

were similar to those used in ours. In vertebrate preparations, there is substantial evidence that RA induces outgrowth via activation of the retinoic acid β receptor. RA, once inside the neuron, binds to ligand-activated nuclear transcription factors that include the retinoic acid receptors (RARs) and/or the retinoid X receptors (RXRs). It is believed that the RARs, specifically RARβ, play a major role in the induction of neurite outgrowth, at least from spinal cord neurons (Corcoran et al., 2002; Dmetrichuk et al., 2005). However, Solomin et al. (1998) recently showed in mice that an RXR may play a role in motor neuron innervation of limbs in vitro. In *Lymnaea*, as in other non-chordates (Kostrouch et al., 1998; Wiens et al., 2003), there is no current evidence for the existence of RARs. We have however recently cloned a full-length cDNA for an RXR from *Lymnaea* (Carter et al., 2005), and like other invertebrate RXRs (de Mendonca et al., 2000; Bouton et al., 2005), this sequence shows a high degree of homology with vertebrate and human RXRs. Though all-*trans* RA (used in this study) binds with 10- to 100-fold higher affinity to RARs than RXRs (Umemiya et al., 1997), it does up-regulate RXR expression in the marine sponge (in addition to exerting developmental morphogenetic effects; Wiens et al., 2003). We have not yet determined whether all-*trans* RA is exerting its effects via the RXR in *Lymnaea* neurons. One possibility is that all-*trans* RA is isomerized to *9*-cis RA (the characteristic ligand for RXR) within the neuron. However, whether this isomerization normally occurs in neurons and what mechanisms might be involved are not yet entirely known (Maden and Hind, 2003). Compared to the existence of RXRs, evidence for the presence of endogenous retinoids in invertebrates is currently scarce. However, retinoids have been detected in the phylogenetically oldest metazoan phylum, the sponges (Bielsalski et al., 1992), and both 9-*cis* and all-*trans* RA are present in the fiddler crab limb blastemas (Hopkins, 2001) as well as the prevertebrate amphioxus (Dalfo et al., 2002). Retinoic acid binding proteins have also been identified in the insect (Mansfield et al., 1998) as well as the shrimp (Gu et al., 2002). Though vitamin A was detected in the pulmonate snail *Helix pomatia* (Eakin and Brandenburger, 1968) and RA can alter eye

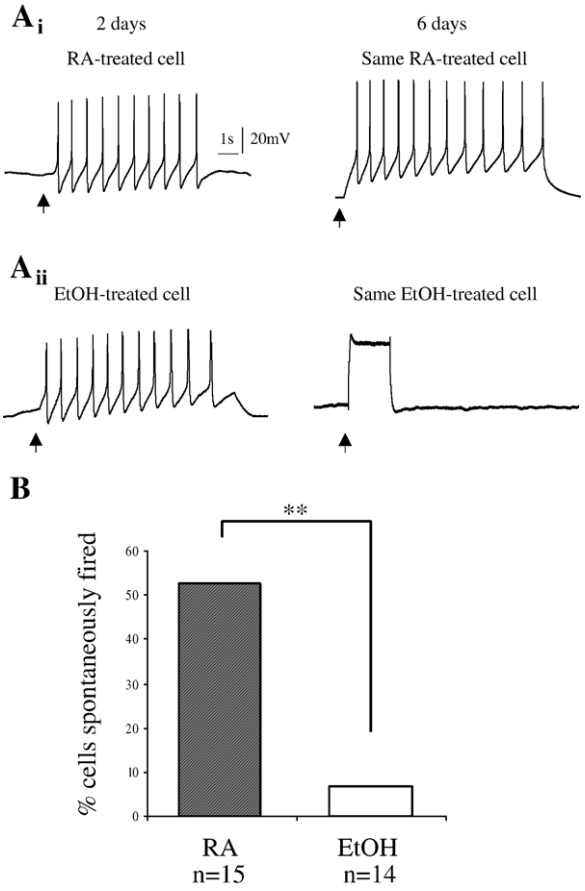

Fig. 6. More cells fired in RA after 6 days of culture. Only cells that fired on day 2 (spontaneous or induced firing) were recorded again on day 6. Representative intracellular electrophysiological recordings of induced firing in a cell cultured in RA (Ai) and a cell cultured in vehicle control (Aii). Following depolarizing current injection, the cell cultured in RA fired action potentials on day 2 and again on day 6. The cell cultured in vehicle control fired action potentials on day 2 but failed to respond to current injection on day 6. Arrowheads indicate the time of current injections. (B) A graph showing that the percentage of cells that spontaneously fired action potentials on day 6 of cell culture was significantly greater in RA than in vehicle control. **$P < 0.01$.

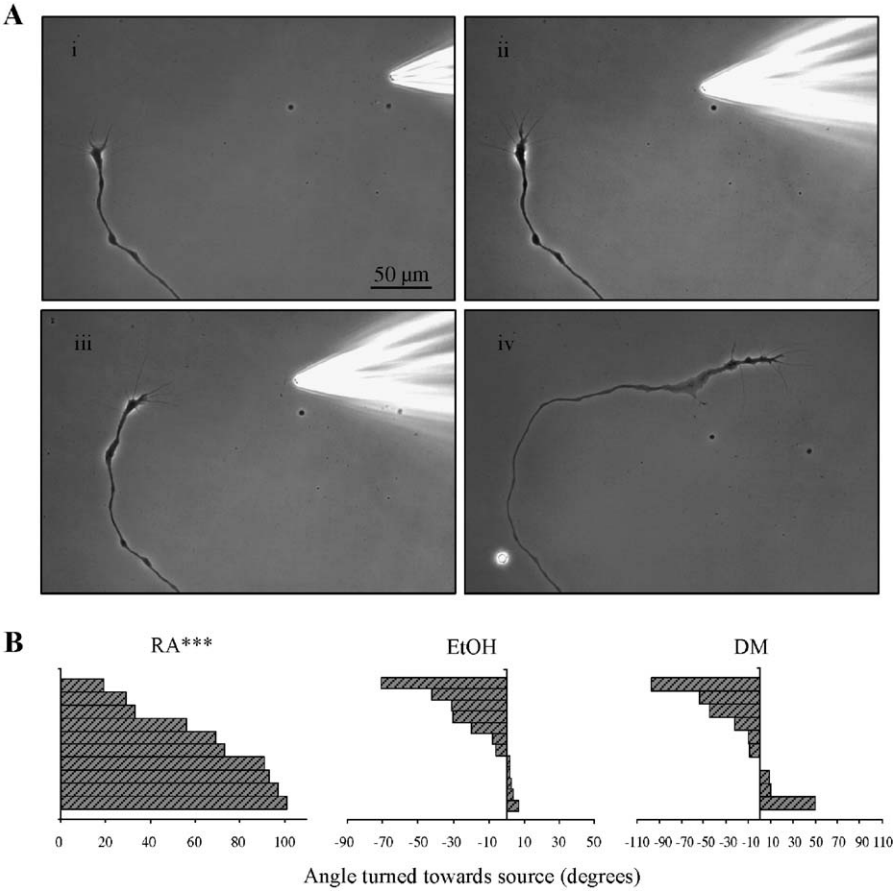

Fig. 7. RA induced positive growth cone turning. (A) A VF cell was cultured in CM and RA (10^{-7} M) for 2 days and then monitored for 1 h to ensure no spontaneous changes in direction occurred. A pipette containing 10^{-5} M RA was positioned about 150 μM from the growth cone and at an angle to the advancing growth cone. (i) Control image prior to the start of RA application. RA was then applied in a pulsatile manner. (ii) Fifteen minutes after the start of RA application. By 2 h (iii), the growth cone had turned toward the source of RA and the RA source was then removed. (iv) At 22 h, even after the removal of the RA source, the growth cone continued to grow in the new direction. (B) Histograms depict the final angle of turning of growth cones exposed to RA (10^{-5} M), EtOH control or DM only control. Each bar on the histogram represents one growth cone from one cell. ***$P < 0.0001$.

formation in *Lymnaea* (Créton et al., 1993), little is known about endogenous retinoids in *Lymnaea* (or other molluscs). However, we have recent evidence from high performance liquid chromatography (HPLC) and mass spectrometry experiments (unpublished observations) that the CNS of adult *Lymnaea* contains RA. Though yet to be tested, it is thus likely that RA exerts effects in vivo, as well as in vitro.

In addition to neurite induction and neuronal survival, some vertebrate studies have also shown that RA directs neurite outgrowth. For example, Maden et al. (1998) showed that embryonic chick neurons grew towards a source of RA (2×10^{-6} M), and we have previously shown that exogenous RA induced directed outgrowth from newt spinal cord (Dmetrichuk et al., 2005). Importantly, such directed outgrowth from the newt spinal cord is also induced by the regeneration blastema (Dmetrichuk et al., 2005), which is thought to both contain and release RA (Brockes, 1992; Scadding and Maden, 1994; Viviano et al., 1995). Here, we demonstrated for the first time that RA can induce turning of a single growth cone toward the source of RA. Furthermore, we demonstrated that, if the source of RA was reversed immediately following induction of the initial turn, the growth cone would turn once again toward the source of RA. It was found that both EtOH and DM application alone to the growth cone induced a slight negative turn of the growth cone. These data suggest that the mechanical pressure (though slight) used to apply the solutions generated a slight repulsive response by the growth cones. Despite this slight negative effect of the mechanical pressure, however, RA was able to induce a much larger positive turning angle. These data are thus consistent with previous vertebrate studies, showing RA-induced chemotaxis, though this is the first known demonstration that RA can induce neurite turning at the level of a single identified growth cone in vitro, in either vertebrate or invertebrate neurons.

As the *Lymnaea* neurons were cultured in both DM and CM in this part of our study, growth cones were exposed in some instances to other (unidentified) neurotrophic factors. We cannot therefore rule out the possibility that some of these other factors may have enabled RA's chemoattractive actions or even acted synergistically with RA. Indeed, it has previously been shown that neurotrophins can act in synergy with RA to induce outgrowth (Corcoran and Maden, 1999; Mey and Rombach, 1999; Plum et al., 2001). However, any other trophic factors present were likely dispersed evenly

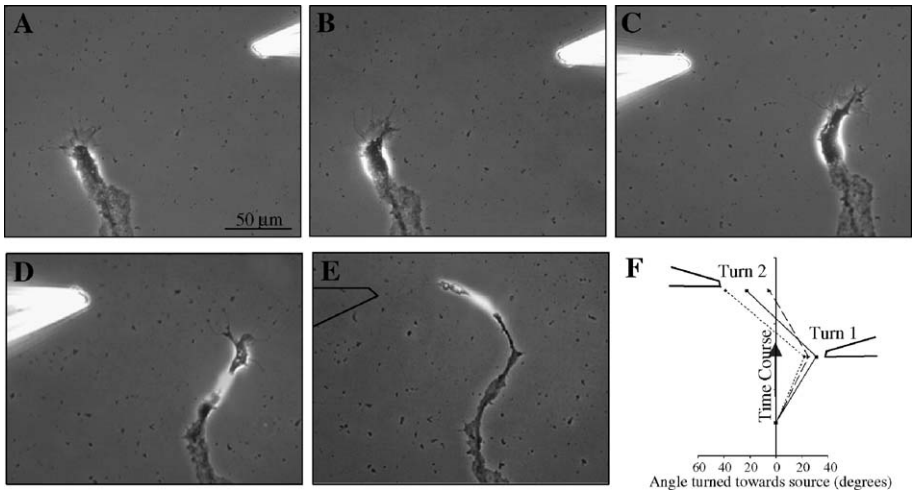

Fig. 8. RA induced zigzagging behavior of *Lymnaea* growth cones. Representative photomicrographs showing that, if the direction of the source of RA was switched from one side of the growth cone to the other, the RA induced a second turning response of the growth cone toward the RA ($n = 3$ of 3). (A–C) RA (10^{-5} M) induced an initial turning response of the growth cone toward RA (B: 20 min of RA application). Following this initial turn, the RA source was re-directed from the opposite side of the growth cone (C: 1.5 h after RA application) and the growth cone responded by turning again toward the source of RA (D–E) (D: 2.5 h after RA applied from opposite side; E: 12 h after last application of RA. (F) Illustration of the first and second turning angles produced by switching the source of RA ($n = 3$).

throughout the media, so their role in inducing growth cone turning is unlikely, though it has been suggested that prior exposure to neurotrophins can affect responses to guidance cues (Cai et al., 1999). However, when our cells were cultured in DM alone, in the absence of any other trophic factors, we observed a similar extent of growth cone turning in response to RA as we did in CM. These data thus strongly suggest that RA is capable of acting independently of other trophic factors to re-direct neurite outgrowth.

One important consideration from this study is the timing of the RA-induced growth cone turning. In most instances, growth cone turning occurred within 1 to 3 h. These data are in agreement with the effects of RA on chick embryonic neurons that grew toward the source of RA after a period of 5 h (Maden et al., 1998). However, in a few cases, the growth cone turning was slower and was only evident around 10 h later. It is unknown at this time how RA is inducing growth cone turning. Though a slow growth cone response may suggest that RA is acting in its classical manner through activation of nuclear transcription factors, there is also precedence in the literature for RA acting in a non-classical manner. For example, Zhang and McMahon (2000) suggest that rapid effects of RA on neuronal gap junctions occur through activation of novel non-nuclear RAR sites. Furthermore, there is evidence to suggest that RA binds directly to protein kinase C (Ochoa et al., 2003), can induce rapid phosphorylation of CREB (Canon et al., 2004) and can induce Ca^{2+} release from intracellular stores via a PLC/PI 3-kinase pathway (Liou et al., 2005), in a nongenomic manner. Even though the effects of RA on growth cone turning in many cases took several hours, we cannot rule out the fact that RA may be acting locally at the neuronal growth cone, as is the case for many other guidance molecules (Campbell and Holt, 2001). Previously, we have shown that the dopamine-induced positive turning of *Lymnaea* growth cones takes up to 2 h (Spencer et al., 2000). Though we did not previously determine whether this dopamine-induced positive turning was mediated locally, we do know that the inhibitory effects of dopamine occur in isolated growth cones (unpublished observations). Thus, it is not implausible that the effects of RA on growth cone turning are mediated locally at the level of the growth cone. As *Lymnaea* neurites can be successfully transected from their cell bodies and continue to grow for several days, this possibility can be explored in future studies.

It has previously been proposed that the failure of many vertebrate CNS neurons to regenerate is largely due to an inhibitory glial environment. However, recent evidence suggests that other signals might play an important role in the regenerative capabilities of central versus peripheral neurons. For example, it has been proposed that neurotrophins may not only stimulate outgrowth but may also enable a neuron to become unresponsive to inhibitory regulators of outgrowth (Cai et al., 1999). RA may prove to be an important factor in the regenerative capabilities of adult central neurons. For example, though RA can induce neurite outgrowth from embryonic mammalian neurons via activation of RARβ, this effect is lost in adult mammalian neurons (due to the absence of RARβ expression). Adult mammalian neurons transfected with RARβ will again respond to RA and regenerate (Corcoran et al., 2002). Furthermore, adult species that retain their ability to regenerate the adult CNS also maintain expression of RARβ in the adult (Dmetrichuk et al., 2005). It is critical to our overall understanding of neuronal regeneration as well as axon guidance during development that we continue to identify genes and molecules that play a role in inducing or guiding outgrowth in the CNS. Our current data support those of others that strongly suggest that RA is an important trophic molecule, though this is the first demonstration that RA enhances viability and induces and

directs outgrowth from invertebrate neurons. This is therefore the first direct evidence that the trophic actions of RA are strongly conserved from invertebrate to vertebrate species.

Acknowledgments

This research was supported by the Banting Research Foundation (Canada) and the Brock University Advancement Fund (BUAF). J.M. Dmetrichuk was supported by a post-graduate scholarship from the Natural Sciences and Engineering Research Council of Canada and by The Premier's Research Excellence Award (Ontario, to G.E.S.). The authors are grateful to Jean Richardson for statistical assistance.

References

Bielsalski, H.K., Doepner, G., Tzimas, G., Gamulin, V., Schoder, H.C., Batel, R., Nau, H., Muller, W.E.G., 1992. Modulation of myb gene expression in sponges by retinoic acid. Oncogene 7, 1765–1774.

Bouton, D., Escriva, H., deMendonca, R.L., Glineur, C., Bertin, B., Noel, C., Robinson-Rechavi, M., de Groot, A., Cornette, J., Laudet, V., Pierce, R.J., 2005. A conserved retinoid X receptor (RXR) from the mollusk *Biomphalaria glabrata* transactivates transcription in the presence of retinoids. J. Mol. Endocrinol. 34, 567–582.

Bradford, P.G., Wang, X., Jin, Y., Hui, P., 1992. Transcriptional regulation and increased functional expression of the inositol triphosphate receptor in retinoic acid-treated HL-60 cells. J. Biol. Chem. 267, 20959–20964.

Brockes, J.P., 1992. Introduction of a retinoid reporter gene into the urodele limb blastema. Proc. Natl. Acad. Sci. U. S. A. 89, 11386–11390.

Cai, D., Shen, Y., De Bellard, M., Tang, S., Filbin, M.T., 1999. Prior exposure to neurotrophins blocks inhibition of axonal regeneration by MAG and myelin via a cAMP-dependent mechanism. Neuron 22, 89–101.

Campbell, D.S., Holt, C.E., 2001. Chemotropic responses of retinal growth cones mediated by rapid local protein synthesis and degradation. Neuron 32, 1013–1026.

Canon, E., Cosgaya, J.M., Scsucova, S., Aranda, A., 2004. Rapid effects of retinoic acid on CREB and ERK phosphorylation in neuronal cells. Mol. Biol. Cell 15, 5583–5592.

Carter, C., Carlone, R., Dmetrichuk, J., Spencer, G., 2005. An RXR nuclear receptor expressed in the CNS of the mollusc *Lymnaea stagnalis*. Genbank Accession #: AY846875.

Corcoran, J., Maden, M., 1999. Nerve growth factor acts via retinoic acid synthesis to stimulate neurite outgrowth. Nat. Neurosci. 2, 307–308.

Corcoran, J., Shroot, B., Pizzey, J., Maden, M., 2000. The role of retinoic acid receptors in neurite outgrowth from different populations of embryonic mouse dorsal root ganglia. J. Cell Sci. 113, 2567–2574.

Corcoran, J., So, P., Barber, R., Vincent, K., Mazarakis, N., Mitrophanous, K., Kingsman, S., Maden, M., 2002. Retinoic acid receptor β2 and neurite outgrowth in the adult mouse spinal cord in vitro. J. Cell Sci. 115, 3779–3786.

Créton, R., Zwaan, G., Dohmen, R., 1993. Specific developmental defects in molluscs after treatment with retinoic acid during gastrulation. Dev. Growth Differ. 35, 357–364.

Dalfo, D., Albalat, R., Molotkov, A., Duester, G., Gonzalez-Duarte, R., 2002. Retinoic acid synthesis in the prevertebrate amphioxus involves retinol oxidation. Dev. Genes Evol. 212, 388–393.

de Mendonca, R.L., Escriva, H., Bouton, D., Zelus, D., Vanacker, J.M., Bonnelye, E., Cornette, J., Pierce, R.J., Laudet, V., 2000. Structural and functional divergence of a nuclear receptor of the RXR family from the trematode parasite *Schistosoma mansoni*. Eur. J. Biochem. 267, 3208–3219.

Dmetrichuk, J.M., Spencer, G.E., Carlone, R.L., 2005. Retinoic acid-dependent attraction of adult spinal cord axons towards regenerating newt limb blastemas in vitro. Dev. Biol. 281, 112–120.

Eakin, R.M., Brandenburger, J.L., 1968. Localization of vitamin A in the eye of a pulmonate snail. Proc. Natl. Acad. Sci. U. S. A. 60, 140–145.

Fukuhara, S., Mukai, H., Munekata, E., 1997. Activin A and all-*trans*-retinoic acid cooperatively enhanced the functional activity of L-type Ca^{2+} channels in the neuroblastoma C1300 cell line. Biochem. Biophys. Res. Commun. 241, 363–368.

Gao, Z.Y., Xu, G., Stwora-Wojczyk, M.M., Matschinsky, F.M., Lee, V.M., Wolf, B.A., 1998. Retinoic acid induction of calcium channel expression in human NT2N neurons. Biochem. Biophys. Res. Commun. 247, 407–413.

Garbe, D.S., Bashaw, G.J., 2004. Axon guidance at the midline: from mutants to mechanisms. Crit. Rev. Biochem. Mol. Biol. 39, 319–341.

Goldberg, J.L., Espinoas, J.S., Xu, Y., Davidson, N., Kovacs, G.T., Barres, B. A., 2002. Retinal ganglion cells do not extend axons by default: promotion by neurotrophic signaling and electrical activity. Neuron 33, 689–702.

Goodman, C.S., 1994. The likeness of being: phylogenetically conserved molecular mechanisms of growth cone guidance. Cell 78, 353–356.

Gu, P.L., Gunawardene, Y.I., Chow, B.C., He, J.G., Chan, S.M., 2002. Characterization of a novel cellular retinoic acid/retinol binding protein from shrimp: expression of the recombinant protein for immunohistochemical detection and binding assay. Gene 288, 77–84.

Gudas, L.J., Sporn, M.B., Roberts, A.B., 1994. Cellular biology and biochemistry of the retinoids. In: Sporn, M.B., Roberts, A.B., Goodman, D.S. (Eds.), The retinoids: biology, chemistry and medicine. Raven, New York.

Hopkins, P., 2001. Limb regeneration in the fiddler crab, *Uca pugilator*: hormonal and growth factor control. Am. Zool. 41, 389–398.

Hunter, K., Maden, M., Summerbell, D., Eriksson, U., Holder, N., 1991. Retinoic acid stimulates neurite outgrowth in the amphibian spinal cord. Proc. Natl. Acad. Sci. U. S. A. 88, 3666–3670.

Kater, S.B., Mills, L.R., 1991. Regulation of growth cone behaviour by calcium. J. Neurosci. 11, 891–899.

Kostrouch, Z., Kostrouchova, M., Love, W., Jannini, E., Piatigorsky, J., Rall, J.E., 1998. Retinoic acid X receptor in the diploblast, *Tripedalia cystophora*. Proc. Natl. Acad. Sci. U. S. A. 95, 13442–13447.

Liou, J.-C., Ho, S.-Y., Shen, M.-R., Liao, Y.-P., Chiu, W.-T., 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.

Maden, M., Hind, M., 2003. Retinoic acid, a regeneration-inducing molecule. Dev. Dyn. 226, 237–244.

Maden, M., Gale, E., Kostetskii, I., Zile, M., 1996. Vitamin A-deficient quail embryos have half a hindbrain and other neural defects. Curr. Biol. 6, 417–426.

Maden, M., Keen, G., Jones, G.E., 1998. Retinoic acid as a chemotactic molecule in neuronal development. Int. J. Dev. Neurosci. 16, 317–322.

Mansfield, S.G., Cammer, S., Alexander, S.C., Muehleisen, D.P., Gray, R.S., Tropsha, A., Bollenbacher, W.E., 1998. Molecular cloning and characterization of an invertebrate cellular retinoic acid binding protein. Proc. Natl. Acad. Sci. U. S. A. 95, 6825–6830.

McFarlane, S., Holt, C.E., 1997. Growth factors: a role in guiding axons? Trends Cell Biol. 7, 424–430.

Mey, J., McCaffery, P., 2004. Retinoic acid signalling in the nervous system of adult vertebrates. Neuroscientist 10, 409–421.

Mey, J., Rombach, N., 1999. Retinoic acid increases BDNF-dependent regeneration of chick retinal ganglion cells in vitro. NeuroReport 10, 3573–3577.

Ochoa, W.F., Torrecillas, A., Fita, I., Verdageur, N., Corbalan-Garcia, S., Gomez-Fernandez, J.C., 2003. Retinoic acid binds to the C2-domain of protein kinase C (alpha). Biochemistry 42 (29), 8774–8779.

Plum, L.A., Parada, L.F., Tsoulfas, P., Clagett-Dame, M., 2001. Retinoic acid combined with neurotrophin-3 enhances the survival and neurite outgrowth of embryonic sympathetic neurons. Exp. Biol. Med. 226, 766–775.

Prince, D.J., Carlone, R.L., 2003. Retinoic acid involvement in the reciprocal

neurotrophic interaction between newt spinal cord and limb blastema in vitro. Dev. Brain Res. 140, 67–73.

Ridgway, R.L., Syed, N.I., Lukowiak, K., Bulloch, A.G., 1991. Nerve growth factor (NGF) induces sprouting of specific neurons of the snail *Lymnaea stagnalis*. J. Neurobiol. 22, 377–390.

Scadding, S.R., Maden, M., 1994. Retinoic acid gradients during limb regeneration. Dev. Biol. 162, 608–617.

Solomin, L., Johansson, C.B., Zetterstrom, R.H., Bissonnette, R.P., Heyman, R.A., Olson, L., Lendahl, U., Frisen, J., Perlmann, T., 1998. Retinoid-X receptor signalling in the developing spinal cord. Nature 395 (6700), 398–402.

Song, H.J., Ming, G.L., Poo, M.M., 1997. cAMP-induced switching in turning direction of nerve growth cones. Nature 388, 275–279.

Spencer, G., Lukowiak, K., Syed, N., 1996. Dopamine regulation of neurite outgrowth from identified *Lymnaea* neurons in culture. Cell. Mol. Neurobiol. 16, 577–589.

Spencer, G., Lukowiak, K., Syed, N., 2000. Transmitter–receptor interaction between growth cones of identified *Lymnaea* neurons determine target cell selection in vitro. J. Neurosci. 20, 8077–8085.

Syed, N., Bulloch, A., Lukowiak, K., 1990. In vitro reconstruction of the respiratory central pattern generator of the mollusc *Lymnaea stagnalis*. Science 250, 282–285.

Syed, N.I., Ridgway, R.L., Lukowiak, K., Bulloch, A.G., 1992. Transplantation and functional integration of an identified respiratory interneuron in *Lymnaea stagnalis*. Neuron 8, 767–774.

Tessier-Lavigne, M., Goodman, C., 1996. The molecular biology of axon guidance. Science 274, 1123–1132.

Tonini, R., Mancinelli, E., Balestrini, M., Mazzanti, M., Martegani, E., Ferroni, A., Sturani, E., Zippel, R., 1999. Expression of Ras-GRF in the SK-N-BE neuroblastoma accelerates retinoic-acid induced neuronal differentiation and increases the functional expression of the IRK1 potassium channel. Eur. J. Neurosci. 11, 959–966.

Toselli, M., Tosetti, P., Taglietti, V., 1996. Functional changes in sodium conductances in the human neuroblastoma cell line SH-SY5Y during in vitro differentiation. J. Neurophysiol. 76, 3920–3927.

Umemiya, H., Fukasawa, H., Ebisawa, M., Eyrolles, L., Kawachi, E., Eisenmann, G., Gronemeyer, H., Hashimoto, Y., Shudo, K., Kagechika, H., 1997. Regulation of retinoidal actions by diazepinylbenzoic acids. Retinoic synergists which activate the RXR-RAR heterodimers. J. Med. Chem. 40, 4222–4234.

Viviano, C.M., Horton, C., Maden, M., Brockes, J.P., 1995. Synthesis and release of 9-*cis* retinoic acid by the urodele wound epidermis. Development 121, 3753–3762.

Wiens, M., Batel, R., Korzhev, M., Muller, W.E.G., 2003. Retinoid X receptor and retinoic acid response in the marine *sponge Suberites domuncula*. J. Exp. Biol. 206, 3261–3271.

Wildering, W.C., Hermann, P.M., Bulloch, A.G., 2001. Lymnaea epidermal growth factor promotes axonal regeneration in CNS organ culture. J. Neurosci. 21 (23), 9345–9354.

Wong, R.G., Hadley, R.D., Kater, S.B., Hauser, G.C., 1981. Neurite outgrowth in molluscan organ and cell cultures: the role of conditioning factor(s). J. Neurosci. 1, 1008–1021.

Wuarin, L., Sidell, N., De Vellis, J., 1990. Retinoids increase perinatal spinal cord neuronal survival and astroglial differentiation. Int. J. Dev. Neurosci. 8, 317–326.

Yamamoto, M., McCaffery, P., Drager, V.C., 1996. Influence of the choroids plexus on cerebellar development: analysis of retinoic acid synthesis. Dev. Brain Res. 93, 182–190.

Zhang, D.Q., McMahon, D.G., 2000. Direct gating by retinoic acid of retinal electrical synapses. Proc. Natl. Acad. Sci. 97, 14754–14759.