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Extensive depolarization and lack of recovery of leech Retzius neurons caused by 2,4 diaminobutyric acid

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ABSTRACT

In this paper we present, for the first time, a detailed account of electrophysiological effects of 2,4-diaminobutyric acid (2,4-DABA). 2,4-DABA is a neurotoxic non-protein amino acid produced by *Cyanobacteria* with a possible link to neurodegenerative disorders in animals and humans. Intracellular recordings were performed on Retzius nerve cells of the leech *Haemopis sanguisuga* using glass microelectrodes filled with 3 mol/L KCl. Our results show that 2,4-DABA is an excitatory amino acid, causing membrane depolarization in a concentration-dependent manner. The most prominent depolarizations of 39.63 ± 2.22 mV and 47.05 ± 4.33 mV, induced by 5×10^{-3} and 10^{-2} mol/L 2,4-DABA respectively, are several times larger than maximal depolarizations induced by either Glutamate, Aspartate, β-N-methylamino-alanine (BMAA) or β-N-oxalylamino-alanine (BOAA) on our model. These 2,4-DABA induced depolarizations evolve through two distinct stages, which is a novel phenomenon in electrical cell activity upon application of an excitatory amino acid, at least on our model. Involvement of two separate mechanisms, suggested by the two stage phenomenon, is discussed in the paper. We also provide evidence that 2,4-DABA induces irreversible functional disturbances in neurons in a concentration-dependent manner, since only half of the cells recovered normal electrical activity after application of 5×10^{-3} mol/L 2,4-DABA, and none recovered after application of 10^{-2} mol/L 2,4-DABA. Effects of both L-2,4-DABA and DL-2,4-DABA were tested and are not significantly different.

1. Introduction

2,4-Diaminobutyric acid (2,4-DABA) is a non-protein amino acid first identified by Catch as a metabolic product of bacteria (Catch et al., 1948). The first report of the neurotoxic properties of 2,4-DABA followed soon (Riggs et al., 1954). In this paper Riggs and associates have applied 2,4-DABA to rats in a dose of 7 mmol/L/kg subcutaneously, which resulted in preconvulsive and convulsive behavior and death. Chronic exposure to 2 mmol/L/kg for 7 days led to little change in behavior, but produced focal morphological degenerative changes most pronounced in the Purkinje cells of the cerebellum. Riggs et al. have concluded that 2,4-DABA has a strong neurotoxic action on rats (Riggs et al., 1954).

These findings have been subsequently confirmed, and additional neurotoxic manifestations, including hyperirritability, weakness in hind legs and tremors in the upper extremities, were observed in rats (Chen et al., 1972; O'Neal et al., 1968) and mice (Ronquist et al., 1980). Aside from being neurotoxic, 2,4-DABA has been shown to be hepatotoxic (O'Neal et al., 1968), and to induce irreversible damage to fibrosarcoma (Ronquist et al., 1980), glioma (Ronquist et al., 1984), and hepatoma (Blind et al., 2003) cells. However, 2,4-DABA has seldom appeared in literature in context of its toxicity, until it was found to be present together with β-N-methylamino-L-alanine (BMAA) in cyanobacterial samples.

BMAA is a neurotoxic amino acid associated with Western Pacific amyotrophic lateral sclerosis – Parkinsonism/dementia complex. Ever since Cox et al. (2003) proposed that *Cyanobacteria* produce BMAA, there has been a continuous effort to detect and quantify BMAA in various matrices. Although the field has been plagued with controversy, the presence of BMAA in aquatic ecosystems is accepted (Faassen, 2014), and BMAA is reported to occur in different geographical locations and environments (Merel et al., 2013).

In many studies searching for BMAA, from the earliest reports (Banack and Cox, 2003) to the most recent ones (Chatziefthimiou et al., 2017), 2,4-DABA was detected together with BMAA, and often attributed a relevant and significant role in environmental toxicity. For instance, analysis of feathers from the carcasses of Lesser Flamingos, during mass mortality events, showed the presence of 2,4-DABA and BMAA in sufficient concentrations for authors to propose that the amino acids could be a principle cause of these mass mortalities. These

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birds consume cyanobacteria and benthic diatoms as a major part of their diet (Metcalf et al., 2013). Masseret et al. report on geographical clusters of patients with ALS surrounding the Thau lagoon, the main shellfish production area of the French Mediterranean coast. As there were no familial cases of ALS in this region the attention was focused on the shellfish, mostly on oysters and mussels, which led to detection of both 2,4-DABA and BMAA in these bivalves and raised concerns for the potential role of dietary exposure to these amino acids in sporadic ALS (Masseret et al., 2013). Furthermore, fish samples from Lake Mascoma, which has a history of cyanobacterial blooms and has been assumed to have spatial links to several sporadic cases of ALS, showed presence of 2,4-DABA and BMAA in the brain tissue (Banack et al., 2015).

Also, in many reports 2,4-DABA was present in higher quantities or in a larger proportion of samples than BMAA. It has also been detected in samples where BMAA could not be confirmed. For example, water samples from shallow pools of the Gobi Desert, where water resources are limited and used by many species for drinking, showed presence of both 2,4-DABA and BMAA, with 2,4-DABA present in concentrations higher than that of BMAA (Craighead et al., 2009). In a study by Kruger et al. BMAA was not detected in any of the 62 cyanobacterial samples of worldwide origin, while 2,4-DABA was detected in 16 (Kruger et al., 2010). Reveillon et al. reported the presence of both 2,4-DABA and BMAA in plankton, peryphyton and mussels in the French Mediterranean coast region. Not only was the concentration of 2,4-DABA in all analysed trophic levels higher than that of BMAA, but 2,4-DABA was present in both bound and free form in all mussel samples collected at any time point during the year (Reveillon et al., 2015). Further investigation of bivalve farming areas on the French coasts showed the presence of 2,4-DABA in higher concentrations than recorded BMAA concentrations in all tested samples of oysters and mussels. Analysis of microalgae, a possible food resource for mollusks, that were isolated from mentioned aquatic areas and cultured, proved existence of 2,4-DABA, but not BMAA (Reveillon et al., 2016). In a study by Glover et al. (2015) in five different commercial natural health products containing *Spirulina*, 2,4-DABA was found in concentrations up to 100-fold higher than BMAA. Fan et al. report that 2,4-DABA was detected in 13 out of 17 strains of *Cyanobacteria* grown in culture, while none of the strains contained BMAA (Fan et al., 2015). 2,4-DABA was also shown to be universally present, with no species-specific or regional differences, in marine bivalve and gastropod mollusks collected along the Chinese coast (Li et al., 2016). The presence of DABA has been shown in the most extreme environments (Cires et al., 2017), and at different trophic levels (Chatziefthimiou et al., 2017).

All these data clearly indicate potential environmental significance of 2,4-DABA. However, despite global presence, proven neurotoxicity and implications in events related to environmental exposure and toxicity, 2,4-DABA has largely been neglected, with most of the attention being directed towards BMAA. This is especially pronounced in electrophysiological studies where, to the best of our knowledge, the effects of 2,4-DABA on membrane potential in context of neurotoxicity are mentioned in only one paper, and even there just as a comparison to BMAA (Weiss et al., 1989).

Having in mind universal presence of 2,4-DABA, its neurotoxic properties, potential significance for aquatic ecosystems and human exposure, as well as lack of electrophysiological data on this amino acid, we have conducted a study to investigate the effects of 2,4-DABA on membrane potential of Retzius nerve cells of the leech *Haemopis sanguisuga*.

2. Materials and methods

Experiments were performed at room temperature (22–25 °C) on Retzius nerve cells of isolated segmental ganglia of the ventral nerve cord of the leech *H. sanguisuga*. The animals were purchased from a local distributor and kept in aquaria in batches of up to 20 leeches per aquarium in (dechlorinated) tap water in a refrigerator at +4 °C. Water was changed twice a week.

The method of dissection has been previously described (Beleslin, 1971) and complies with ethical guidelines issued by the institution's and national ethical boards. Briefly, the leeches were first anaesthetized with 10% ethanol. Then, the ventral nerve cord with its enveloping blood sinus was removed from the animal in short segments of three ganglia via a ventral longitudinal incision. Removed segments were immediately transferred to a 2.5 ml plastic chamber with leech Ringer and fixed by means of fine steel clips. The blood sinus was then dissected away. No further preparation was performed since Nicholls and Kuffler (1964) have shown that compounds added to the bathing medium can easily reach the cells without further dissection. The plastic chamber was then placed in grounded Faraday's cage mounted on a fixed table in a manner that prevents vibrations. Identification and penetration of the cells was performed in the cage under a stereomicroscope. The Retzius neurons were identified by their position in the ganglion, their size (60–80 μm in diameter) and by their bioelectrical properties: resting membrane potential of −30 to −60 mV and spontaneous activity of 1 action potential per second (Sawyer, 1986).

Prior to the experiments the chamber was flushed with fresh Ringer solution, microelectrode dipped into the solution and allowed 20–30 min for equilibration. The cells were penetrated using a micromanipulator (Leitz, Germany) under a stereomicroscope, and allowed to stabilize after penetration.

To perform the experiments content of the chamber was completely replaced by 2,4-diaminobutyric acid solution of desired concentration. To change the solution, the chamber was continuously flushed with a volume of fluid 4 times that of the chamber volume. The perfusion rate was such that impaled microelectrode remained inside the cell during and after the perfusion, and was usually completed in 10–15 s. The cells were kept in 2,4-DABA solution for 1 or 3 min, depending on the experiment, and then the chamber was flushed with Ringer solution again.

2.1. Electrophysiological recordings

The membrane potential was recorded using standard single-barreled glass microelectrodes. Micropipettes were pulled from thick wall capillaries with internal filament (outside diameter 1.5 mm, inside diameter 0.84 mm, World Precision Instruments, USA) on a vertical puller (PE-6, Narishige, Japan) and then filled with 3 mol/L KCl shortly after being pulled. The tip diameter of the electrodes was 1 μm, tip potentials were less than 5 mV, and the microelectrode resistance was 20–25 MΩ in standard Ringer solution. Only experiments in which the microelectrode drift was no larger than 2 mV were used.

The recordings were amplified using high input impedance amplifier (model 1090, Winston Electronics, USA). Microelectrodes were connected to the amplifier via an Ag–AgCl wire. The ground electrode was an Ag–AgCl pellet in a separate chamber filled with Ringer solution connected to the experimental chamber by a 3 mol/L KCl 3% agar bridge. The recordings were displayed on a two-channel oscilloscope (HM 205-3, Hameg, Germany) and permanently recorded on a pen recorder (L 7025 II, Linseis, Germany).

2.2. Solutions

The leech Ringer solution was of the following composition (in mmol/L): NaCl 115.5, KCl 4, $CaCl_2$ 2, NaH_2PO_4 0.3, Na_2HPO_4 1.2 (pH = 7.2). DL-2,4-diaminobutyric acid and L-2,4-diaminobutyric acid (both by Sigma–Aldrich) were kept in concentrated aqueous stock solutions. Adequate concentrations of the amino acids were prepared by pipetting appropriate amounts of the stock solution to the Ringer solution just before use.

2.3. Data analysis

All results are expressed as means ± S.E.M. with n indicating number of trials. Comparison between mean values was made with a two-tailed Student's *t*-test and one-way ANOVA. The normality of data was tested using normality Shapiro–Wilk and Kolmogorov–Sminov tests. *p*-Values of less than 0.05 were considered significant.

3. Results

3.1. Effects of DL-2,4-DABA on membrane potential

Our initial goal was to investigate the effects of DL-2,4-DABA on Retzius neurons' membrane potential. Since there is little literature data on the electrophysiological effects of 2,4-DABA, we have followed an established protocol used previously in our laboratory to test for the effects of other amino acids – both environmental, such as β-N-oxalylamino-L-alanine (BOAA) and β-N-methylamino-L-alanine (BMAA), and endogenous to the leech, such as glutamic and aspartic acid. As most of these amino acids have shown first visible effects on the membrane potential at the concentration of 10^{-3} mol/L in our previous experiments (Lopicic et al., 2009b), we have chosen to use that concentration first for DL-2,4-DABA as well. Concentration of 10^{-3} mol/L DL-2,4-DABA produced a minute but statistically significant membrane potential depolarization of 2.36 ± 0.48 mV ($p < 0.01$, $n = 8$). The membrane potential did not hyperpolarize after washout with standard Ringer solution and spontaneous firing of action potentials was maintained throughout the application (Fig. 1A).

We have proceeded by increasing this initial concentration 3, 5, and 10-fold. Application of 3×10^{-3} mol/L DL-2,4-DABA led to a depolarization of the resting membrane potential by 11.42 ± 0.94 mV ($p < 0.01$, $n = 6$). During the peak of the depolarization spontaneous activity of the cells was abolished. The depolarization was followed by a transient hyperpolarization after washout with leech Ringer solution (Fig. 1B). Concentration of 5×10^{-3} mol/L induced rapid and substantial depolarization of membrane potential from resting level of − 47.82 ± 3.27 mV to − 8.20 ± 2.55 mV, i.e. by 39.63 ± 2.22 mV ($p < 0.01$, $n = 8$) with cessation of spontaneous activity, and followed by a transient hyperpolarization upon washout with leech Ringer solution (Fig. 1C). Finally, application of 10^{-2} mol/L DL-2,4-DABA depolarized the resting membrane potential by 47.05 ± 4.33 mV, from − 50.10 ± 2.26 mV to − 3.04 ± 5.19 mV ($p < 0.01$, $n = 6$). Spontaneous activity stopped early during application. The repolarization phase after washout with leech Ringer solution was terminated before reaching the resting level, and the membrane potential was maintained at this new depolarized level for the duration of the recording (Fig. 1D). The data are summarized in Table 1.

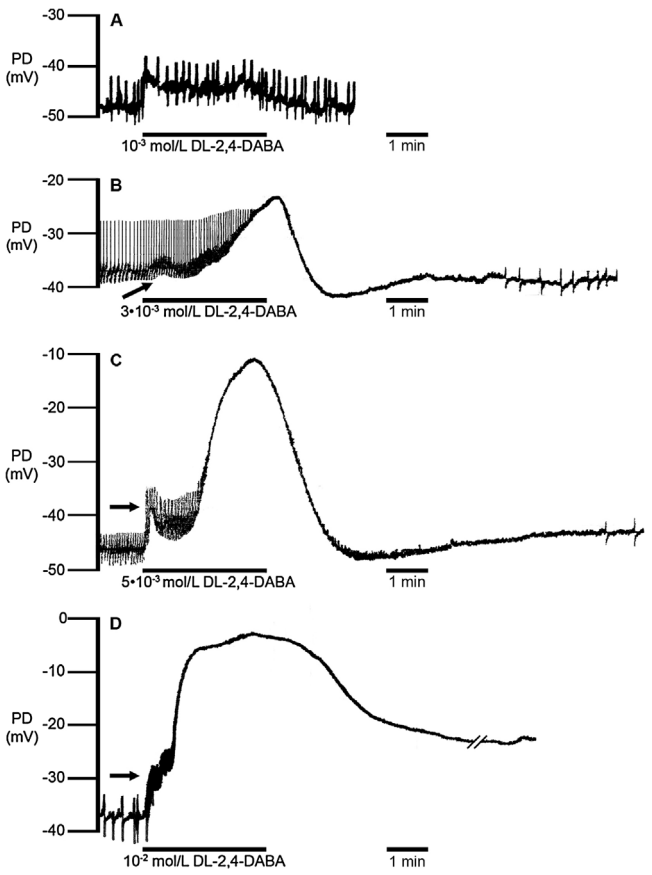

Fig. 1. Effects of 10^{-3} mol/L (A), 3×10^{-3} mol/L (B), 5×10^{-3} mol/L (C) and 10^{-2} mol/L (D) DL-2,4-DABA on membrane potential (PD) of Retzius nerve cells. At the break in D (//) a 10 min period of stable recording was omitted; arrows indicate first stage of depolarization.

We have used ANOVA with post hoc Tukey test to check for differences between effects of individual concentration groups. All concentration combinations show statistically significant difference of effects, except for the difference between depolarizations induced by 5×10^{-3} and 10^{-2} mol/L which is not significantly different ($p > 0.05$).

The results clearly indicate concentration dependency of the effect, so we have fitted a dose-response curve to the data (solid line in Fig. 2A). The calculated maximum of the effect is 47.08 ± 0.03 mV, and the half maximal effect is produced at the concentration of (4.0 ± 0.2) × 10^{-3} mol/L.

The most remarkable feature of the effects described above are large depolarizations exceeding 30 mV elicited by 5×10^{-3} mol/L and 10^{-2} mol/L DL-2,4-DABA. Depolarizations as substantial as those have never before been recorded in our experiments with other excitatory amino acids on the same model.

3.2. Comparison of effects of DL-2,4-DABA with effects of other excitatory amino acids previously tested in our laboratory

To compare effects of DL-2,4-DABA on membrane potential with those of other neurotoxic excitatory amino acids tested on our model, namely glutamic acid, aspartic acid, BOAA and BMAA, we have used data from our previous experiments. To facilitate the comparison we provide here a previously unpublished recording of an 11 mV depolarization induced by 10^{-2} mol/L L-BMAA (Fig. 3), which is a representative example of effects induced by these amino acids. For other recordings and detailed data please refer to (Lopicic et al., 2009a,b).

Fig. 2B shows dose response curves for glutamic acid, aspartic acid, BOAA, BMAA, and DL-2,4-DABA. From Fig. 2B and numerical data presented in Table 2. it is clear that, although not the most potent, DL-2,4-DABA is by far the most effective of the excitatory amino acids.

DL-2,4-DABA is different to other excitatory amino acids in two other important ways - its effect consists of two distinct stages and there is lack of membrane potential recovery after application of higher concentrations of the amino acid. These effects are presented below.

3.3. The two stage nature of the DL-2,4-DABA effect

While analyzing the recordings we have noticed that concentrations of 3×10^{-3} mol/L DL-2,4-DABA and higher produce an effect that consists of two stages. The first stage (arrows in Fig. 1B–D.) is a smaller, shorter lasting depolarization which is followed by a larger depolarization described in the first section of the results. As indicated before, the first stage did not appear in any of the cells treated with 10^{-3} mol/L DABA. Application of 3×10^{-3} mol/L DL-2,4-DABA produced the first stage in 3 out of 6 cells with an average amplitude of 1.67 ± 0.26 mV. This initial depolarization, when present, was followed by a spontaneous membrane potential repolarization to the resting (control) level,

Table 1
Effects of DL-2,4-DABA on cell membrane potential of leech Retzius nerve cells.

Conc. (mol/L)	Membrane potential (mV)		Depolarization (mV)	p	n
	Control	DL-2,4-DABA			
10^{-3}	−47.44 ± 2.90	−45.07 ± 2.95	2.36 ± 0.48	< 0.01	8
3×10^{-3}	−43.56 ± 2.86	−32.14 ± 2.60	11.42 ± 0.94	< 0.01	6
5×10^{-3}	−47.82 ± 3.27	−8.20 ± 2.55	39.63 ± 2.22	< 0.01	8
10^{-2}	−50.10 ± 2.26	−3.04 ± 5.19	47.05 ± 4.33	< 0.01	6

Data shown as means ± S.E.M., Conc. – applied concentration of DL-2,4-DABA, *p* – t-test significance level relative to control, *n* – number of trials.

after which the second stage started (Fig. 1B). Concentrations of 5×10^{-3} and 10^{-2} mol/L DL-2,4-DABA induced the first stage in all cells, with average amplitudes of 7.91 ± 0.81 mV and 14.37 ± 0.79 mV respectively. When 5×10^{-3} mol/L DL-2,4-DABA was applied the first stage was followed by a spontaneous membrane potential repolarization prior to the second stage, but this repolarization was incomplete as it never reached the resting membrane potential level (Fig. 1C). The first stage during application of 10^{-2} mol/L DL-2,4-DABA was immediately followed by the second depolarization, i.e. there was no membrane potential recovery between the two stages (Fig. 1D). The difference of the first stage depolarization between individual concentration groups was highly significant for all group pairs ($p < 0.01$, ANOVA with post hoc Tukey test). In temporal terms the first stage was rather uniform and lasted for up to 40 s irrespective of the DL-2,4-DABA concentration that was applied. The data are summarized in Table 3 (data presented only for cells that have exhibited the first stage).

Since the first stage depolarizations also show concentration dependency, we have fitted a dose-response curve to these data as well (dashed line in Fig. 2A.). The calculated maximum of the first stage effect is 14.41 ± 0.18 mV, and the half maximal effect is produced at the concentration of $(5.0 \pm 0.1) \times 10^{-3}$ mol/L.

It is interesting to note that the calculated maximal effect of the first stage fits very well with the maximal effects of other excitatory amino acids presented in Table 2. It is the second stage that stands out in terms of magnitude of depolarization. It is also noteworthy that none of the excitatory amino acids that we have tested previously had shown the first stage effect even at highest concentrations that we had applied.

3.4. Lack of recovery

Another striking characteristic of the effect of DL-2,4-DABA was

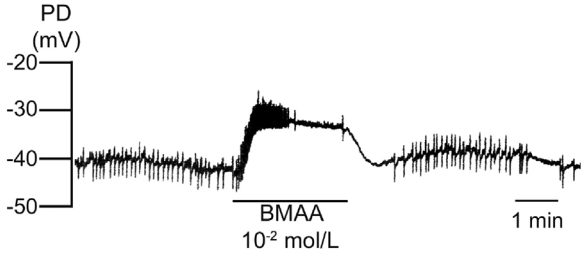

Fig. 3. Effect of 10^{-2} mol/L β-N-methylamino-L-alanine (BMAA) on membrane potential (PD) of Retzius nerve cells.

significant variation of recovery when the cells were returned to standard leech Ringer solution after application of the amino acid.

All concentrations of either glutamate, aspartate, BOAA or BMAA that were applied in our previous experiments produced effects that were followed by full recovery in all of the treated cells after 1–5 min. It was the same for DL-2,4-DABA at concentrations of 10^{-3} and 3×10^{-3} mol/L. However, when 5×10^{-3} mol/L of the substance was applied only half of the cells recovered. In cells that did recover, the recovery was incomplete, delayed or both. There was a complete lack of recovery when 10^{-2} mol/L DL-2,4-DABA was applied, i.e. none of the cells treated with this concentration had recovered even after more than 30 min of continuous registration (Fig. 1D).

Lack of recovery in this context means that the membrane potential was maintained at levels less negative than the resting membrane potential and that the spontaneous firing of the action potentials did not resume. By incomplete recovery we mean that either the membrane potential was maintained at levels less negative then the resting membrane potential but spontaneous firing of action potentials was

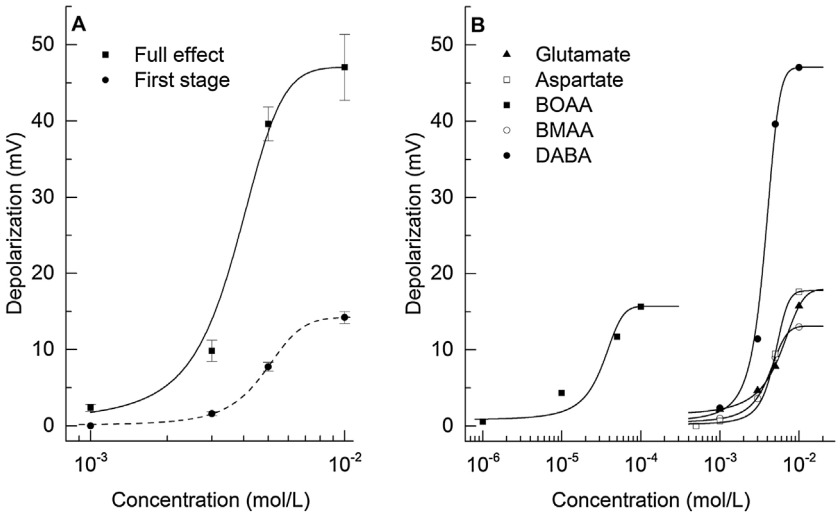

Fig. 2. Dose-dependency of depolarizing action of DL-2,4-DABA on leech Retzius nerve cells (A) and comparison of effects of DL-2,4-DABA with other excitatory amino acids on cell membrane potential (B). All concentrations applied for 3 min. BOAA-β-N-oxalylamino-L-alanine, BMAA-β-N-methylamino-L-alanine. In B error bars omitted for reasons of clarity.

Table 2
Comparison of effects of endogenous and exogenous excitatory amino acids on membrane potential of Retzius nerve cells.

	L-glutamate	L-aspartate	L-BOAA	L-BMAA	DL-2,4-DABA
Dep_{max} (mV)	17.93 ± 0.53	17.74 ± 0.39	15.70 ± 0.91	13.10 ± 0.03	47.08 ± 0.03
EC_{50} (mol/L)	$6.0 \pm 0.2 \times 10^{-3}$	$5.0 \pm 0.1 \times 10^{-3}$	$3.0 \pm 0.9 \times 10^{-5}$	$4.0 \pm 0.9 \times 10^{-3}$	$4.0 \pm 0.2 \times 10^{-3}$

Data shown as means ± S.E.M.. Dep_{max} – maximal depolarization of cell membrane potential, EC_{50} – concentration inducing half maximal effect. L-BOAA –β-N-oxalylamino-L-alanine, L-BMAA –β-N-methylamino-L-alanine.

Table 3
First stage depolarization elicited by DL-2,4-DABA on leech Retzius nerve cells.

Conc. (mol/L)	Control MP (mV)	MP of the first stage (mV)	First stage depolarization (mV)	*p*	*n*
3×10^{-3}	−41.63 ± 1.80	−39.96 ± 1.80	1.67 ± 0.26	< 0.05	3
5×10^{-3}	−47.82 ± 3.27	−39.91 ± 2.77	7.91 ± 0.81	< 0.01	8
10^{-2}	−50.10 ± 2.26	−35.72 ± 1.68	14.37 ± 0.79	< 0.01	6

Data shown as means ± S.E.M., Conc. – applied concentration of DL-2,4-DABA, MP – membrane potential, *p* – t-test significance level relative to control, *n* – number of trials.

restored, or that the membrane potential recovered to the resting level but spontaneous firing of action potentials did not resume. We considered the recovery to be delayed when it took the cells more than five minutes to reestablish the resting membrane potential and/or spontaneous firing of action potentials.Delayed recovery can be seen in Fig. 1C.

As mentioned before, due to lack of electrophysiological data on the effects of DL-2,4-DABA we have initially used our standard experimental protocol which involves bath application of a substance for 3 min. Since DL-2,4-DABA produced lack of recovery under these conditions we decided to test whether the shortening of application would improve recovery. For this we have chosen the concentration of 5×10^{-3} mol/L because 10^{-2} mol/L induces such a pronounced depolarization that, in our experience, the shortening of the application was unlikely to amend recovery.

The application of 5×10^{-3} mol/L DL-2,4-DABA for 1 min was followed by a full recovery in all treated cells within 5 min of washout with standard Ringer solution. Other effects were the same as when the substance was applied for 3 min – both stages were present, and neither the first stage nor the overall depolarization were significantly different than those during the 3 min application (Table 4, Fig. 4A).

3.5. Comparison of DL-2,4-DABA and L-2,4-DABA

Having in mind that it is L-2,4-DABA rather than DL-2,4-DABA that is routinely detected in *Cyanobacteria* specimens, and that there were some reports of different actions of the L- and D-isomers of DABA (for details see Discussion), we have decided to test the effects of L-2,4-DABA as well. For this comparison we have chosen to apply both L- and DL-2,4-DABA in concentration of 5×10^{-3} mol/L, as it is the concentration at which DL-2,4-DABA produces depolarization larger than 30 mV, setting it apart from other excitatory amino acids, but also allows for full recovery if the administration time is reduced to 1 min. We took advantage of this, because full recovery after 1 min application enabled us to apply both isomers consecutively on the same cell. To avoid the possible effects of subsequent applications of DABA (for example enhancement or reduction of effect in the second application) we have alternated the order of application of the isomers – in some cells L-2,4-DABA was applied first, while in others it was DL-2,4-DABA. Also, the second isomer was applied only if we had at least 20 min of stable recording after full recovery of the first application.

Table 4
Comparison of the effects of 5×10^{-3} mol/L DL-2,4-DABA and L-2,4-DABA on cell membrane potential of leech Retzius nerve cells. Substances applied for 1 min.

Substance	Contorl MP (mV)	1st stage (mV)	2nd stage (mV)	*n*
DL-2,4-DABA	−47.82 ± 3.27	7.75 ± 0.59 ($p^{*} > 0.05$)	39.63 ± 2.22 ($p^{*} > 0.05$)	8
L-2,4-DABA	−48.31 ± 2.34	8.69 ± 1.49 ($p^{**} > 0.05$)	38.64 ± 3.57 ($p^{**} > 0.05$)	7

Data shown as means ± S.E.M., MP membrane potential, 1st stage – first stage depolarization, 2nd stage – overall (second stage) depolarization, p^{*} – t-test significance level relative to 5×10^{-3} mol/L DL-2,4-DABA applied for 3 min, p^{**} – t-test significance level relative to 5×10^{-3} DL-2,4-DABA applied for 1 min.

In concentration of 5×10^{-3} mol/L L-2,4-DABA, applied for 1 min, produced depolarization of the cell membrane potential by 38.64 ± 3.57 mV ($p < 0.01$, $n = 7$) during which there was a complete cessation of spontaneous activity (Fig. 4B.). Both stages were present, and neither the first stage nor the overall depolarization were significantly different between the two isomers (Table 4, Fig. 4B).

4. Discussion

In this paper we provide, for the first time, a detailed account of electrophysiological effects of 2,4-diaminobutyric acid on membrane potential of nerve cells.

2,4-Diaminobutyric acid (2,4-DABA) is a neurotoxic and hepatotoxic non-protein amino acid. It is a major component of polymyxin group of antibiotics (Catch et al., 1948) and a component of bacterial cell walls (Perkins and Cummins, 1964), present in several *Lathyrus* species (Ressler, 1964; Vanetten and Miller, 1963), mammalian brain (Nakajima et al., 1967), *Cyanobacteria* (Kruger et al., 2010), crustaceans (Banack et al., 2014), fish, aquatic plants and water reservoirs (Al-Sammak et al., 2014). It is the presence in *Cyanobacteria* and water environment that has rekindled the interest in 2,4-DABA in recent years, because of *Cyanobacteria* ubiquity and a proposed link between *Cyanobacteria* and neurotoxic effects in animals and humans (Li et al., 2016; Main and Rodgers, 2017). There is, to the best of our knowledge, lack of electrophysiological in vitro studies of 2,4-DABA.

Our results show that 2,4-DABA is an excitatory amino-acid, as it led to membrane potential depolarization in a concentration dependent manner. This excitatory effect is in concert with hyperirritability, tremor and convulsions, reported in virtually all investigations concerning neurotoxicity of 2,4-DABA in living animals (Beart and Bilal, 1977; Chen et al., 1972; Johnston and Twitchin, 1977; O'Neal et al., 1968; Ressler et al., 1961; Riggs et al., 1954; Ronquist et al., 1980). This is also in agreement with the results of Weiss et al. The primary goal of their investigation was the electrophysiological effect of BMAA on murine cortical neurons in culture, but they have also tested 2,4-DABA as a structural isomer to BMAA by applying it in a single concentration

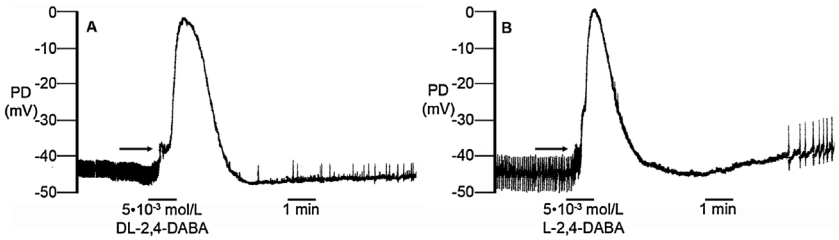

Fig. 4. Effects of 5×10^{-3} mol/L DL-2,4-DABA (A) and 5×10^{-3} mol/L L-2,4-DABA (B) applied for 1 min on membrane potential (PD) of Retzius nerve cells. Arrows indicate first stage of depolarization.

of 1.5 mM. This concentration produced a minor depolarization of the membrane potential but the authors, unfortunately, did not provide numerical data for this effect(Weiss et al., 1989).

Other excitatory amino acids that are accepted or postulated to be environmental neurotoxins, like Domoic acid, BOAA and BMAA, have been shown to induce neurotoxicity through the mechanism of excitotoxicity. In excitotoxicity nerve cell death is preceded by membrane potential depolarization of unusual intensity, duration, or both, which triggers a chain of events that ultimately leads to nerve cell destruction. In this context, substantial depolarization elicited by 2,4-DABA reported in this paper implies a significant capacity of the amino acid to trigger excitotoxicity. We have also shown that 2,4-DABA is considerably more effective on our model than BOAA, BMAA or Glutamate.

The neurotoxic potential of 2,4-DABA is further substantiated by lack of recovery present in our experiments at 2,4-DABA concentrations of 5×10^{-3} and 10^{-2} mol/L, which demonstrates irreversible functional disturbance induced by this amino acid. This corresponds to reports of 2,4-DABA neurotoxicity, as Ronquist et al. have shown that 5 mM 2,4-DABA does not kill all of the exposed cells, while 10 mM does (Ronquist et al., 1980).

The depolarizing effect of 2,4-DABA in concentrations of 3×10^{-3} mol/L and higher in our experiments evolved through two distinct stages, which is a novel phenomenon in electrical cell activity upon application of an excitatory amino acid, at least on our model. The two stage dynamics of the depolarizing effect is of unknown origin, but it might indicate that two separate mechanisms are involved in electrophysiological effects of 2,4-DABA. Weiss et al. (1989) have provided evidence that 2,4-DABA induces depolarization of neurons via activation of glutamate receptors (GluR), while Christensen and Ronquist have shown that 2,4-DABA uptake into the nerve cells involves neutral amino acid transport system A (TSA) (Christensen and Ronquist, 1992). TSA is Na^+ dependent and electrogenic, i.e. activation of the transporter leads to influx of Na^+ into the cell and depolarization of the membrane potential. In our experiments, the first stage of 2,4-DABA induced depolarization is strikingly similar to effects of other excitatory amino-acids, and the dose-dependent curve constructed for the first stage alone corresponds well to the dose-dependent curves for other excitatory amino acids tested on our model. The second stage, on the other hand, shows a pronounced depolarization, much higher than any elicited by other excitatory amino acids. Excessive activation of TSA upon 2,4-DABA application could lead to massive influx of Na^+ and subsequently to pronounced depolarization, similar to the second stage of the 2,4-DABA effect. We, therefore, propose that two separate mechanisms are engaged in 2,4-DABA induced depolarization – the first being glutamate receptor mediated, while the second could be mediated by activation of neutral amino acid transport system A. We are currently investigating this hypotheses, and although there are other processes that could be responsible for the effects reported here, our initial results seem to support the proposed GluR/TSA mechanism.

Chen et al. have shown that classical effects of 2,4-DABA intoxication in rats (hyperirritability, tremor and convulsions) are present when L-2,4-DABA is applied intraperitoneally, but do not occur when D-2,4-DABA is administered via the same route. However, when applied intracisternally, both induced the same effects (Chen et al., 1972). Our results confirm that DL-2,4-DABA and L-2,4-DABA produce the same effects when applied directly to the nervous tissue. Moreover, our results provide further evidence that 2,4-DABA has direct effect on neurons, a position that was initially disputed (O'Neal et al., 1968).

The route of administration used in this paper, direct action of 2,4-DABA on nerve cells, illustrates a plausible exposure scenario that can occur in natural environments, as 2,4-DABA was detected in rat brains after subcutaneous injections (Vivanco et al., 1966), in mouse central nervous system after injections in the tail vein (Appelgren et al., 1982), and was also detected in brains of fish living in lakes with frequent cyanobacterial blooms (Banack et al., 2015). The relevance of the concentrations used in this paper is hard to assess in terms of realistic natural exposure levels. The concentrations of 2,4-DABA reported in literature vary significantly from tens of ng/g (Chatziefthimiou et al., 2017) to hundreds of μg/g (Bishop et al., 2018). More importantly, extrapolation of in vitro and ex vivo findings to environmental effects on animal and human health requires precise information not only on environmental occurrence, but also knowledge on potential bioaccumulation, bioavailability, metabolism and the elimination. To this date there is little knowledge of these information for 2,4-DABA. In light of our data we propose that further and more vigorous investigation into these and other properties of 2,4-DABA is necessary.

5. Conclusion

We conclude that 2,4-DABA is a potent excitatory amino-acid which, in leech Retzius neurons, at concentrations of 5×10^{-3} mol/L and 10^{-2} mol/L, permanently alters the functional properties of nerve cells. DL-2,4-DABA, when applied directly to neurons, exhibits the same effects as L-DABA. Due to its global presence and possible effects on water ecosystems as well as risk for human exposure environmental occurrence, potential bioaccumulation, bioavailability, metabolism, elimination, toxicity and mechanisms of action of 2,4-DABA warrant further investigation.

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