TITLE

Physiological control of bioluminescence in a deep-sea planktonic worm, *Tomopteris helgolandica* Greeff, 1879

RUNNING TITLE

Tomopteris' bioluminescence control

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SUMMARY

Tomopteris helgolandica Greeff 1879 (Tomopteridae) is a transparent holoplanktonic polychaete that can emit a bright light. In this work, we investigated the emission pattern and control of this deep-sea worm's luminescence.

Potassium chloride depolarisation applied on anesthetised specimens triggered a maximal yellow light emission from specific parapodial sites, suggesting that a nervous control pathway was involved. A pharmacological screening revealed a sensitivity to carbachol, which was confirmed by a dose-light response associated with a change in the light emission pattern, where physiological carbachol concentrations induced flashes and higher concentrations induced glows. The light response induced by its hydrolysable agonist, acetylcholine, was significantly weaker but was facilitated by eserine pretreatment. In addition, a specific inhibitory effect of tubocurarine was observed on carbachol-induced emission. Lastly, KCl- and carbachol-induced light responses were significantly reduced when preparations were pre-incubated in Ca^{2+}-free artificial sea water or in different calcium channel blockers (verapamil, diltiazem) and calmodulin inhibitor (trifluoperazine) solutions. All of these

results strongly suggest that *T. helgolandica* produces its light flashes *via* activating nicotinic cholinergic receptors and a calcium-dependent intracellular mechanism involving L-type calcium channels.

INTRODUCTION

Planktonic organisms can achieve almost perfect invisibility *via* transparency, making it one of the most valuable and fascinating adaptations to pelagic environments (Johnsen and Widder, 1998). Paradoxically, at first sight, some of these cryptic organisms are also brightly luminous (Haddock and Case, 1999; Poupin *et al.*, 1999).

The tomopterid holoplanktonic polychaetes belong to a particularly diverse family that is extremely transparent (Johnsen and Widder, 1998) and include at least 11 bioluminescent species (Poupin *et al.*, 1999). Although the spectral distribution has been measured in only two species (λ_{max}= 565 nm for *Tomopteris nisseni* Rosa, 1908 and λ_{max}= 570 nm for *T. septentrionalis* Quatrefages, 1865) (Dales, 1971; Latz *et al.*, 1988), these pelagic worms are often described as yellow emitters as opposed to most bioluminescent marine organisms that emit a blue-green light. Additionally, given the species-specific luminous organ distribution observed in this family, this unusual luminescence has been suggested to be an intraspecific communication signal (Harvey, 1952; Dales, 1971; Latz *et al.*, 1988). However, since the review of Dales (1971), this elegant hypothesis is often highlighted despite a lack of evidence because basic experimental data are missing such as emission pattern and associated physiological control mechanisms. Here, we focused on these fundamental aspects and presented a pharmacological approach of the luminescence control of *T. helgolandica* Greeff, 1879, a widespread, previously established bioluminescent East Atlantic species (Harvey, 1952).

MATERIAL AND METHODS

Organisms' collection

T. helgolandica specimens (1.5-6.0 cm in length), were collected from October 2010 to November 2012 at a 200-300 m depth from two connected fjords, Raunefjorden and Korsfjorden (Western Norway), using two types of towed net samplers: the Isaacs-Kidd midwater trawl (1.75 m wide x 1.30

m high mouth, 6.5 m long, 500 µm mesh aperture) or ring plankton nets (1.5 m diameter mouth, 300 µm mesh aperture) depending on the weather and the vessel's equipment. Live specimens were housed in classical aquariums or in kreisel tanks commonly used for maintaining gelatinous zooplankton (Baker, 1963; Raskoff *et al.*, 2003) in a permanent dark cold room (6-8°C) at the Espegrend Marine Biological Station of the University of Bergen.

Anesthesia

Several anesthetics that were previously used on polychaetes (Smaldon and Lee, 1979; Costa-Paiva *et al.*, 2007; Ross and Ross, 2008; Cooper, 2011) including magnesium chloride, propylene phenoxetol and tricaine mesylate were unsuccessful with *T. helgolandica*. However, preliminary experiments demonstrated that menthol efficiently anesthetised the animals within 30 min and reduced the interindividual variability of the emitted light compared to non-treated specimens without affecting light emission parameters. Thus, before each experiment, the organisms were relaxed for 30 min in a menthol solution applied at increasing concentrations (0.25 - 2.5 g l^{-1}).

Pharmacology

The organisms were dissected from the head to the tail into serial preparations that comprised three parapod pairs. Each preparation was placed into 50 µl of artificial sea water (400.4 mmol l^{-1} NaCl, 9.6 mmol l^{-1} KCl, 52.3 mmol l^{-1} $MgCl_2$, 9.9 mmol l^{-1} $CaCl_2$, 27.7 mmol l^{-1} Na_2SO_4, 20 mmol l^{-1} Tris, final pH 8.3). Next, light production was triggered by adding 50 µl of a given test solution. For each studied specimen, one preparation was treated with the control stimulus (200 mmol l^{-1} KCl or, depending on the tested effect and based on preliminary results, 1 mmol l^{-1} cholinergic agonist) whereas the other specimens were treated with different pharmacological substances (Table 1). The light responses were standardised and expressed as a percentage of the control light response. All of the experiments were designed following the Latin square principle: from one specimen to another, the preparation from an identical position is never treated twice by the same solution to eliminate possible interindividual or interpreparation variability. The pharmacological solutions were prepared in artificial sea water buffered at pH 8.3 just before the experiments were performed. The light intensity was measured for

10-20 min with a single tube luminometer (FB12, 2005, Berthold technologies). To avoid light stress or artefactual measurements, all handling and experiments were performed in partial darkness or under red lighting.

Statistics

All of the statistical analyses were performed with JMP software (v 10.0.0, 2012, SAS Institute Inc.) on log-transformed data ($\sum\log(x_{(1\rightarrow n)})/n$) for relative values > 1. Variance normality and equality were previously tested by the Shapiro-Wilk test and Levene's test, respectively. When these parametric assumptions were not met, a one-way ANOVA was replaced by a non-parametric Kruskal-Wallis ANOVA to assess the significant difference between more than two groups. All of the pairwise comparisons were tested using a *post hoc* Student's t-test (each pair), Dunnett's test (with control) or a *post hoc* Dunn's test, as appropriate. Each difference was considered to be significant at 0.05. For clarity, the graphically illustrated values are expressed as the geometric means ($\sqrt{(x_1*x_2*\ldots*x_n)}$) with the corresponding s.e.m..

RESULTS

Light emission pattern

When applied on whole specimens, potassium chloride (200 mmol l^{-1} KCl) triggered a maximal yellow light emission at specific parapodial sites and locally reached 10^3 Mq s^{-1} (Fig. 1A, C). Due to a linear relationship between the total emitted light (L_{tot}) and the maximal light intensity (L_{max}) of the control light responses (Fig. 1B), only the L_{tot} is presented.

Screening of neurotransmitters

The primary neurotransmitter families known to mediate bioluminescence throughout invertebrates were pharmacologically screened on *T. helgolandica's* parapods (Nicol, 1960; Case and Strause, 1978; Anctil, 1979; Gardner and Walker, 1982; Anctil, 1987; Walker *et al.*, 1996) (Table 2). Only carbachol elicited a luminescence higher than the ASW-induced emission (mechanical stimulus).

112

113 **Extrinsic cholinergic control**

114 The amount of emitted light increased with increasing carbachol concentrations (Fig. 3A), and
115 different emission patterns were observed at various concentrations. At low carbachol concentrations,
116 the pattern consisted of a series of weak intensity flashes (Fig. 2A, top left panel), which was in
117 contrast with the monophasic shape of 1 mmol l^{-1} carbachol light emission (Fig. 2A, top right panel)
118 that is similar to a KCl-induced light response (Fig. 1A). However, the tissue preparations generally
119 reponded poorly to acetylcholine compared to its non-hydrolysable agonist, carbachol. Thus, an
120 eserine pretreatment was tested. This cholinesterase inhibitor induced a weak light response (0.04 ±
121 0.03 % of KCl) and significantly facilitated acetylcholine-induced emission but did not affect
122 carbachol-induced light emission (Fig. 2B). Lastly, the specificity of the cholinergic receptors was
123 evaluated using nicotinic and muscarinic blocking agents, tubocurarine and atropine, respectively
124 (Table 1). Only tubocurarine significantly inhibited carbachol-induced emission (Fig. 2C). This
125 observation was confirmed with the nicotinic agonist DMPP which triggered an intense light emission
126 (9519.55 ± 8490.38 % of KCl).

127

128 **Intrinsic control: calcium requirement**

129 Given that the nicotinic control pathway suggests that Ca^{2+} is a second messenger, we aimed to
130 investigate the calcium-dependence of the reaction. Preincubating the tissue preparations in Ca^{2+}-free
131 artificial sea water significantly inhibited the KCl- and carbachol-induced luminescence responses by
132 95 and 100 %, respectively (Fig. 3A). The luminescence was also significantly reduced by different
133 calcium channel blockers, verapamil (phenylalkylamines) and diltiazem (benzothiazepine), and by
134 trifluoperazine, a calmodulin inhibitor (Fig. 3B).

135

136 **DISCUSSION**

137 Although some bioluminescent organisms produce a continuous glow, most light emission signals are
138 transient events mediated by specific control mechanisms (Nicol, 1960). Two control levels are
139 commonly distinguished: an extrinsic control represented by peripheral control pathways and an

intrinsic control that includes the photogenic reaction and the related intracellular signalling pathways (Case and Strause, 1978). In self-luminescent metazoans characterised by differentiated photogenic structures, emission is either controlled by hormones (Claes and Mallefet, 2009) or *via* coupling mechanisms between photocytes and excitable cells, including neural, muscular or epithelial cells (Herring and Morin, 1978; Anctil, 1987; Hastings and Morin, 1991; Krönström *et al*., 2009). Although luminescence can originate in a nerve-free bioluminescent epithelium, such as in the conducting epithelia of some Hydrozoa (Bassot *et al.*, 1978; Dunlap *et al.*, 1987) and Anthozoa (Germain and Anctil, 1996), it is most frequently controlled by neural pathways (Nicol, 1960; Case and Strause, 1978). In addition to turning the light emission on and off, nervous control abilities can modulate and adjust the intensity, duration, frequency or angular distribution of a light signal and thus generate diversity and specificity. However, a large proportion of the functional diversity of the existing emission patterns and control systems is unknown, especially in annelids, where the most detailed bioluminescence control studies have been limited to polynoid and chaetopterid benthic species (Gardner and Walker, 1982; Anctil, 1987). Therefore, the luminescence control of pelagic species worms has been poorly documented (Harvey, 1952; Haddock *et al.*, 2010).

According to our results, *T. helgolandica*‘*s* luminescence is under nervous control, as revealed by yellow luminescence induced by KCl depolarisation in nervous fibres, which directly or indirectly causes a photogenic structure response (De Bremaeker *et al.*, 1996). Furthermore, the pharmacological screen revealed a dose-dependent carbachol sensitivity. In fact, carbachol and acetylcholine both induced light emission, but the tissue preparations demonstrated a low responsiveness to acetylcholine. Pharmacological carbachol concentrations (over 0.1 mmol l^{-1}) elicited a monophasic signal, similar to KCl-induced light emission, but acetylcholine 1 mmol l^{-1} failed to elicit such a pattern. However, an eserine pretreatment significantly facilitated acetylcholine-induced emission, which attained intensities emitted by low carbachol concentrations (< 0.1mmol l^{-1}, nearest physiological concentrations) and suggested an involvement of cholinesterase activity. Lastly, the flash trains (L_{max} = 400 Mq s^{-1}) observed at the lowest concentrations were likely more representative of the naturally expressed signal. The specific inhibitory effect of tubocurarine on carbachol-induced emission not only indicates an involvement of the cholinergic pathway but also demonstrates the nicotinic receptor prevalence.

These observations were comforted by the sensitivity of the samples to the nicotinic agonist DMPP and by their calcium-dependent light response which suggested that L-type calcium channels were also involved. *T. helgolandica* produces yellow flashes from each parapod through neural control that activates nicotinic cholinergic receptors and a calcium-dependent intrinsic mechanism.

Numerous and widespread cholinergic control mechanisms exist in annelids (Gardner and Walker, 1982; Walker *et al.*, 1996). Their control of bioluminescence is relatively well established in Polynoidae and Chaetopteridae (Nicolas *et al*., 1978; Gardner and Walker, 1982; Anctil, 1987) and is reinforced by the present study of one Tomopteridae. However, because of its ubiquity, the specific mode, level and site of action of acetylcholine in the bioluminescent process remain unclear. Muscarinic cholinergic and serotoninergic mechanisms have been described in benthic scale-worms (Polynoïdae) as part of the excitatory pathway of elytral luminescence (Nicol, 1954; Nicolas *et al*., 1978; Miron *et al*., 1987; Anctil *et al*., 1989). The tube-worm *Chaetopterus variopedatus* (Chaetopteridae) produces a glowing blue mucus in response to the contractile action of the adjacent epithelio-muscular cells, which are controlled by muscarinic cholinergic and GABAergic pathways (Nicol, 1952; Anctil, 1981; Martin and Anctil, 1984; Anctil, 1987). Given that some photocytes in others organisms are not directly innervated but are controlled by adjacent supportive cells that trigger light emission by epithelial conduction (Anctil, 1987; Dunlap *et al.*, 1987), the calcium entry *via* L-type channels we observed could act at both the neuro-photocyte level and an intermediate level. The presence parapod nerve fibres, revealed by the histological studies of Greeff (1882; 1885) and Bonhomme (1952) on *T. mariana* and *T. keferteini*, respectively, suggested that the bioluminescence of these worms was under nervous control. In particular, Greeff exhibited a scheme of photogenic organs with direct nerve connections. However, the characterisation of photogenic structures remains ambiguous (Malaquin and Carin, 1922; Bonhomme, 1952).

Despite the differences observed in the light emission pattern, their control and the bioluminescence characteristics between polynoid and chaetopterid worms, the luminescence have been associated with defensive functions. The same hypothesis has been suggested for the blue luminescence of the benthic polychaete *Polycirrus perplexus* (Terebellidae) (Huber *et al.*, 1989) and for the ‘green bombs’ expelled by recently described deep-sea pelagic specimens belongings to Acrocirridae, (Osborn *et al.*,

2011). Lastly, only syllid worms, whose behaviour has been well-studied, use their green light emission for both deterrence and intraspecific communication during mating swarms (Wilkens and Wolken, 1981; Tsuji and Hill, 1983; Fischer and Fischer, 1995; Gaston and Hall, 2000; Deheyn and Latz, 2009). Given that the open ocean does not facilitate contact between planktonic organisms, an atypical emission wavelength would be highly advantageous for Tomopteridae. Although the emission of yellow light has been interpreted by numerous authors as a specific signal that involves a private communication channel (Harvey, 1952; Dales, 1971; Latz *et al.*, 1988), the maximal wavelength of *T. septentrionalis* (λ_{max} = 570 nm) does not match its spectral sensitivity, which is centred on blue (Buskey and Swift, 1985).

Nevertheless, it is likely that *T. helgolandica*'s yellow light may play different roles, as suggested by the observation of different emission patterns - flash against glow - according to the stimulus applied. Flash is often associated with a deterrent function, whereas glows are considered attractive, suggesting that the worm modulate the light output depending on the context that incites bioluminescence use. However, in the absence of further experimental data, this hypothesis remains speculative.

CONCLUSION

Our results strongly support the hypothesis that *T. helgolandica*'s bioluminescence is under nervous control, revealing new insight into the pathways involved. The yellow light flashes are produced *via* activation of nicotinic cholinergic receptors and a calcium-dependent intracellular mechanism involving L-type calcium channels.

However, the understanding of tomopterids' bioluminescence at an ecological level is beyond our current knowledge. In addition to studying the intrinsic mechanisms of light emission, an assessment of the mechanisms that govern their visual capabilities as well as their reproductive biology and behaviour will be performed in our future research.

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LEGENDS

drug			concentration
usual name	commercial name	pharmacology	(mmol l^{-1})
KCl	potassium chloride	depolarizing agent	200
serotonin	5-hydroxytryptamine	serotoninergic neurotransmitter	1
epinephrine	(±)-epinephrine	adrenergic neurotransmitter	1
GABA	gamma-aminobutyric acid	GABAergic neurotransmitter and neuromodulator	1
nitric oxide	sodium nitroprusside	guanylyl cyclase activator	1
carbachol	carbamoylcholine chloride	cholinergic agonist	1
DMPP	1,1-dimethyl-4-phenyl-piperazinium iodide acetylcholine	nicotinic agonist	1
acetylcholine	chloride	cholinergic neurotransmitter	1
eserine	eserine	cholinesterase inhibitor	1
tubocurarine	d-tubocurarine chloride	cholinergic nicotinic receptor antagonist	1
atropine	atropine sulfate	cholinergic muscarinic receptor antagonist	1
nifedipine	nifedipine	calcium channel blocker	1
diltiazem	diltiazem chloride	calcium channel blocker	1
trifluoperazine	trifluoperazine dihydrochloride	calmodulin inhibitor	1
verapamil	(±)-verapamil	calcium channel blocker	1

Table 1. Detailed list of chemical and pharmacological substances (Sigma-Aldrich Co.) used in experiments aimed at assessing the nervous control of luminescence in *Tomopteris helgolandica*.

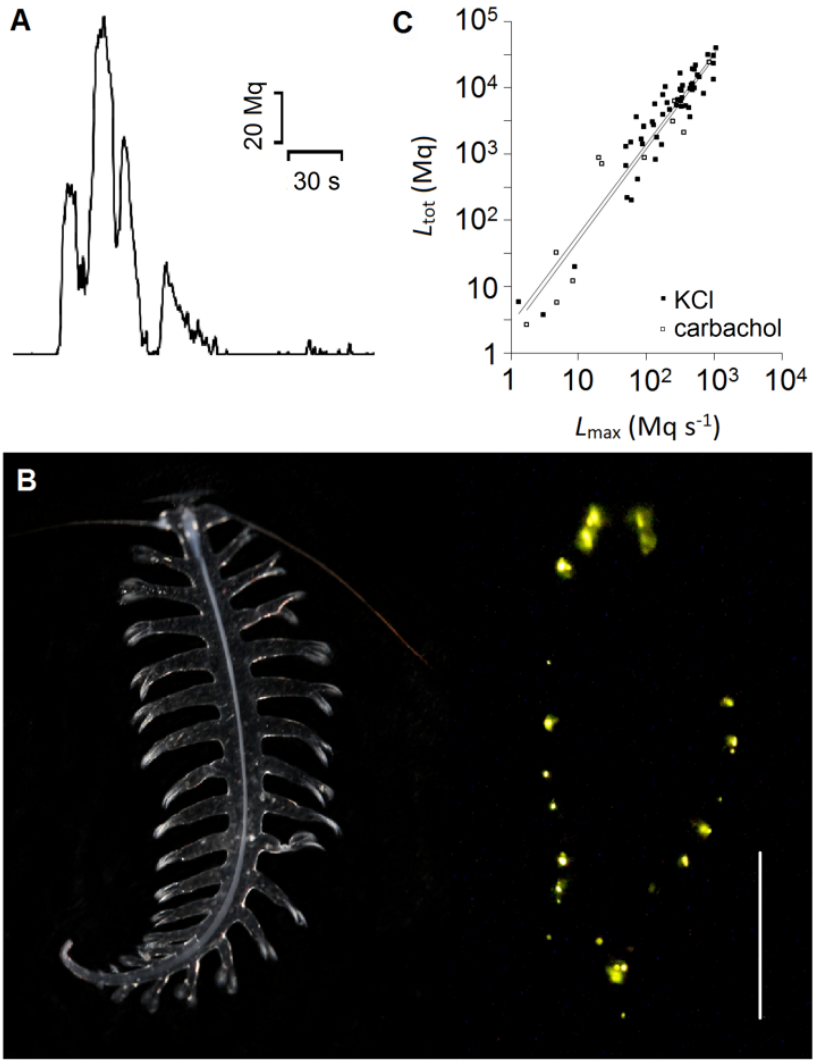

Fig. 1. Light emission pattern of *T. helgolandica*.

(A) Typical curve shape of maximal KCl-induced light emission. (B) *T. helgolandica* was photographed in natural light and its KCl-induced bioluminescence was photographed in the dark. Scale bar, 1 cm. (C) Linear relationship between the total quantity of light emitted (L_{tot}) and the maximal light intensity (L_{max}) for 200 mmol l^{-1} KCl- and 1 mmol l^{-1} carbachol-induced luminescence. Mq = megaquanta = 10^6 photons. R^2-values are 0.8944 and 0.8822 respectively, and the slopes (p = 0.6921) and intercepts (p = 0.6609) are significantly equal.

drug	L_{tot} (% KCl)	p-value	n
ASW (control)	4.67 ± 4.22		5
serotonin	16.56 ± 15.13	1.000	5
epinephrine	53.71 ± 52.76	1.000	5
GABA	15.76 ± 10.60	1.000	5
nitric oxide	89.40 ± 79.55	1.000	4
carbachol	1129.88 ± 482.90	0,2839	5

Table 2. Screening of neurotransmitters' effects on isolated tissue preparations of *T. helgolandica*. The light intensities are expressed as a function of KCl-induced luminescence.

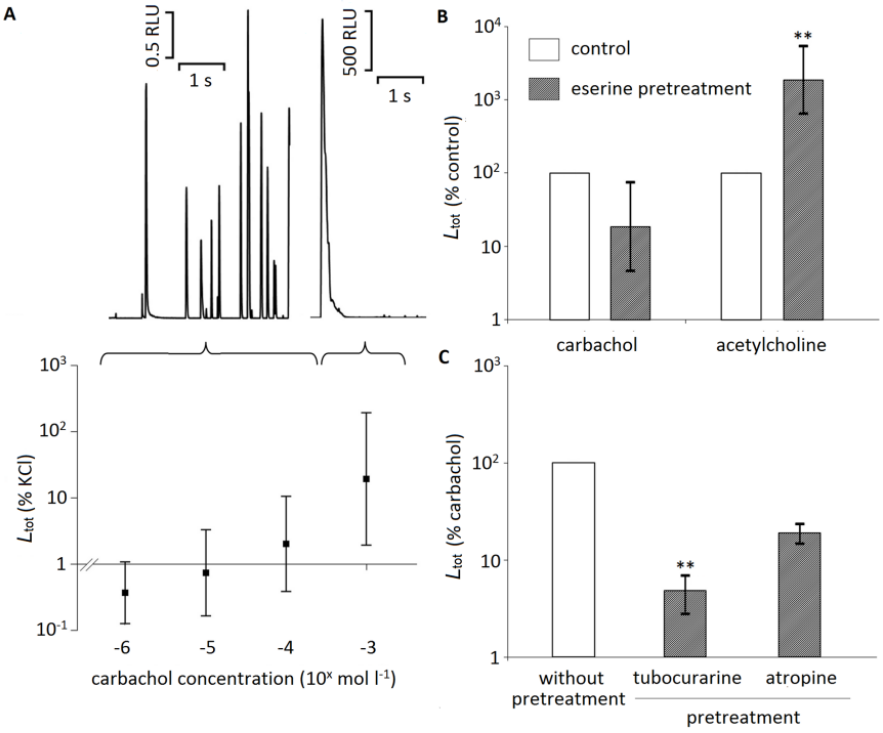

Fig. 2. Physiological control of bioluminescence in *T. helgolandica*.

(A) Carbachol dose-light response – the total quantity of light emitted during the experiment (L_{tot}) is expressed as a percentage of the value obtained with KCl application (theoretical physiological maximum) – and parapod luminescent response patterns in response to low (top left) and high (top right) carbachol concentrations. (B) Effect of an eserine pre-treatment on the luminescence induced by carbachol and acetylcholine. The values represent the L_{tot} increase expressed as a percentage of each

activator injected alone (without eserine pre-treatment). Eserine significantly increased the luminescent response to acetylcholine but did not affect carbachol-induced luminescence. (C) The effect of atropine and tubocurarine pretreatments on carbachol-induced luminescence. Tubocurarine significantly decreased the luminescent response to carbachol, but atropine did not significantly affect this luminescence. (n=6; ** p<0.01).

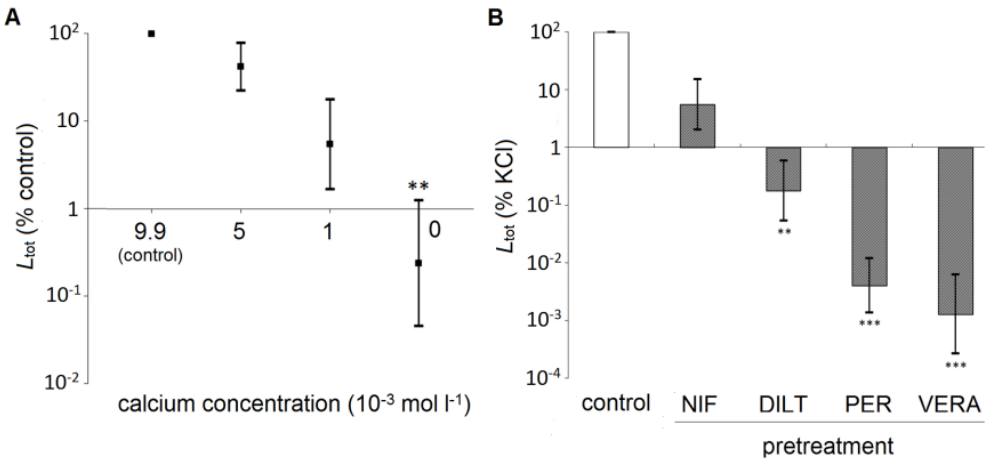

Fig. 3. Intrinsic control of bioluminescence in *T. helgolandica*.

(A) Dose-dependent inhibitory effect of calcium depletion. The values represent the L_{tot} decrease expressed as a percentage of those obtained in complete artificial sea water (10 mmol l^{-1}). (B) The effect of calcium organic inhibitors (NIF = nifedipine, DILT = diltiazem, PER = trifluoperazine, VERA = verapamil) on KCl-induced luminescence. (n=6; * p<0.05, ** p<0.01, *** p<0.001).

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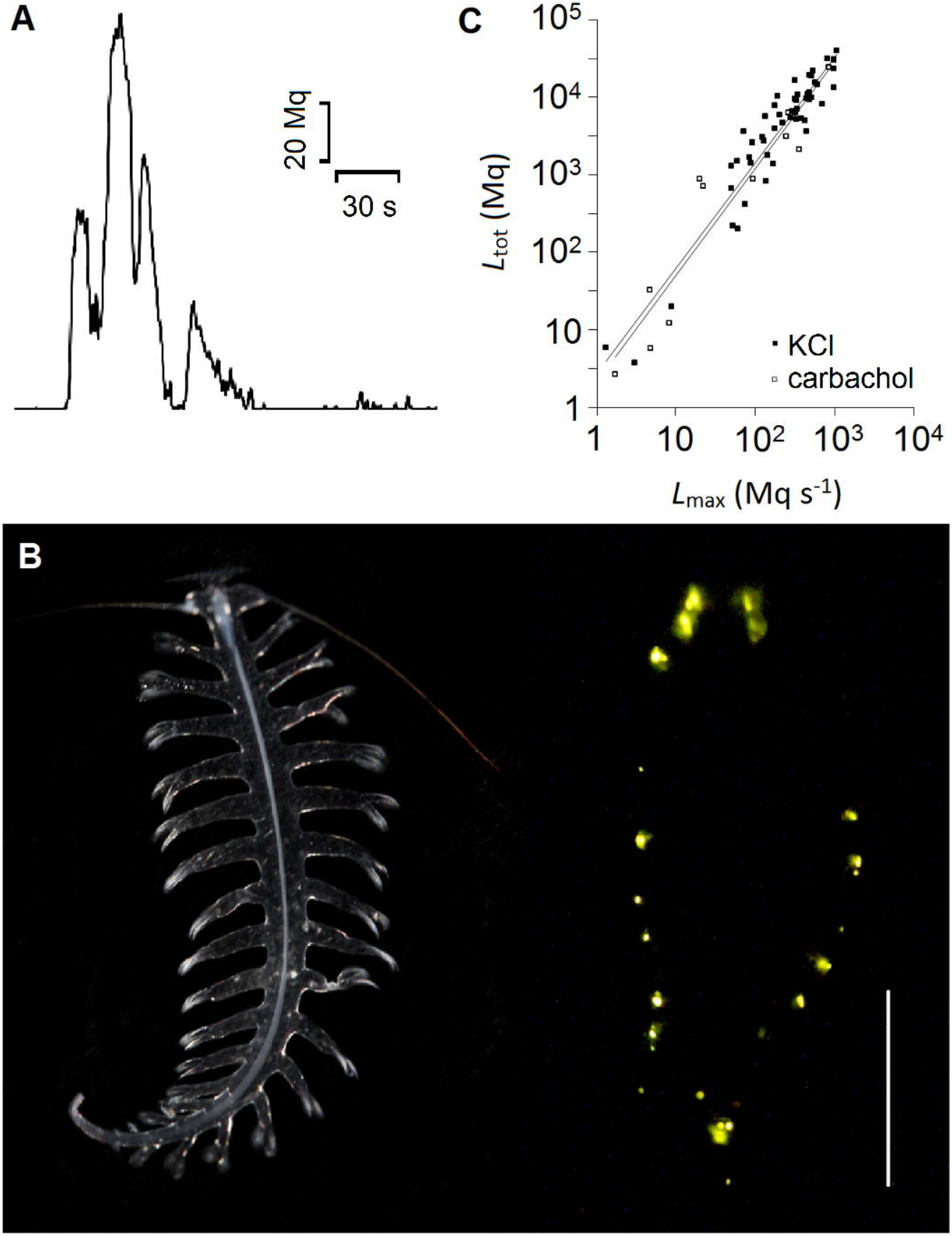
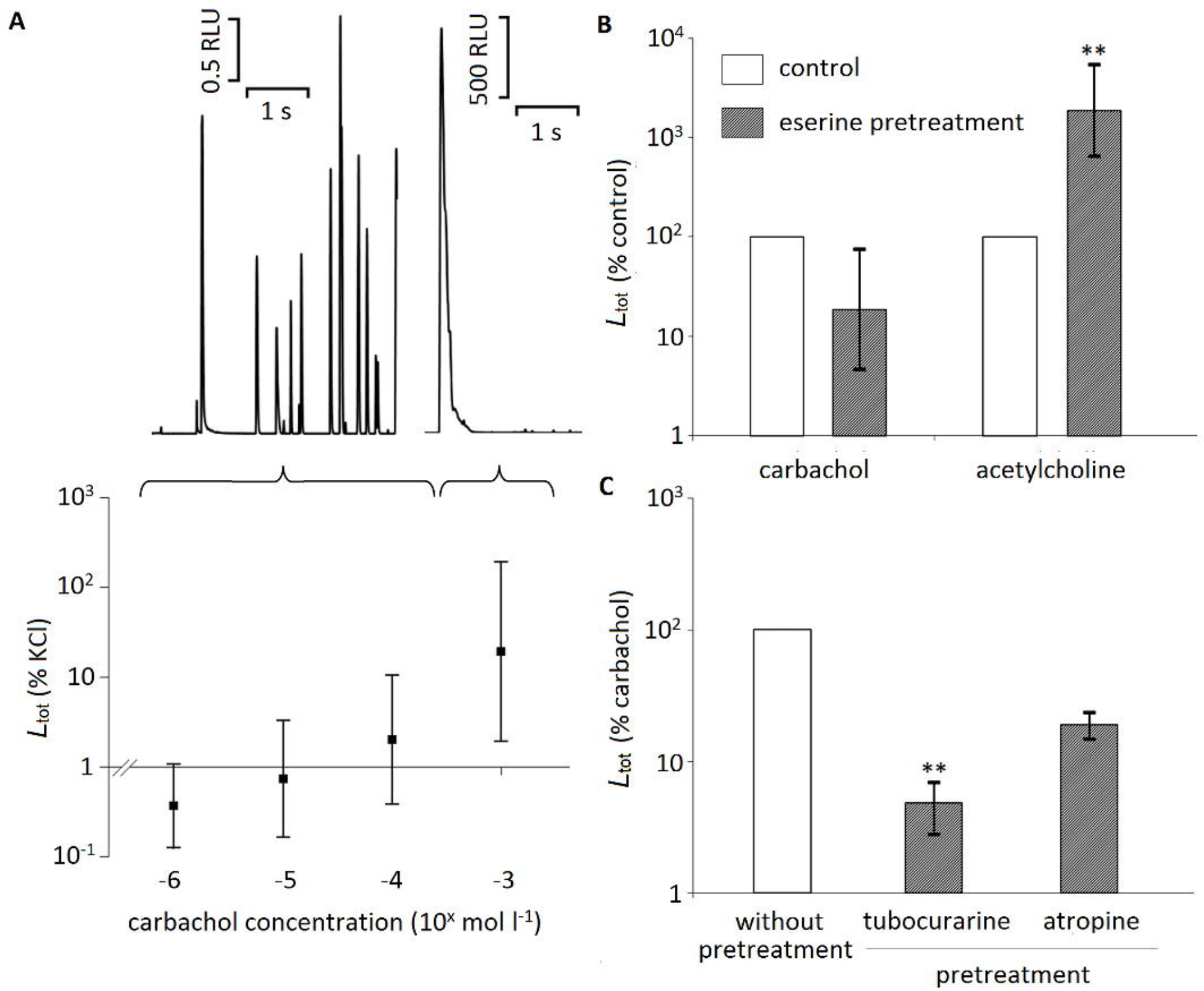
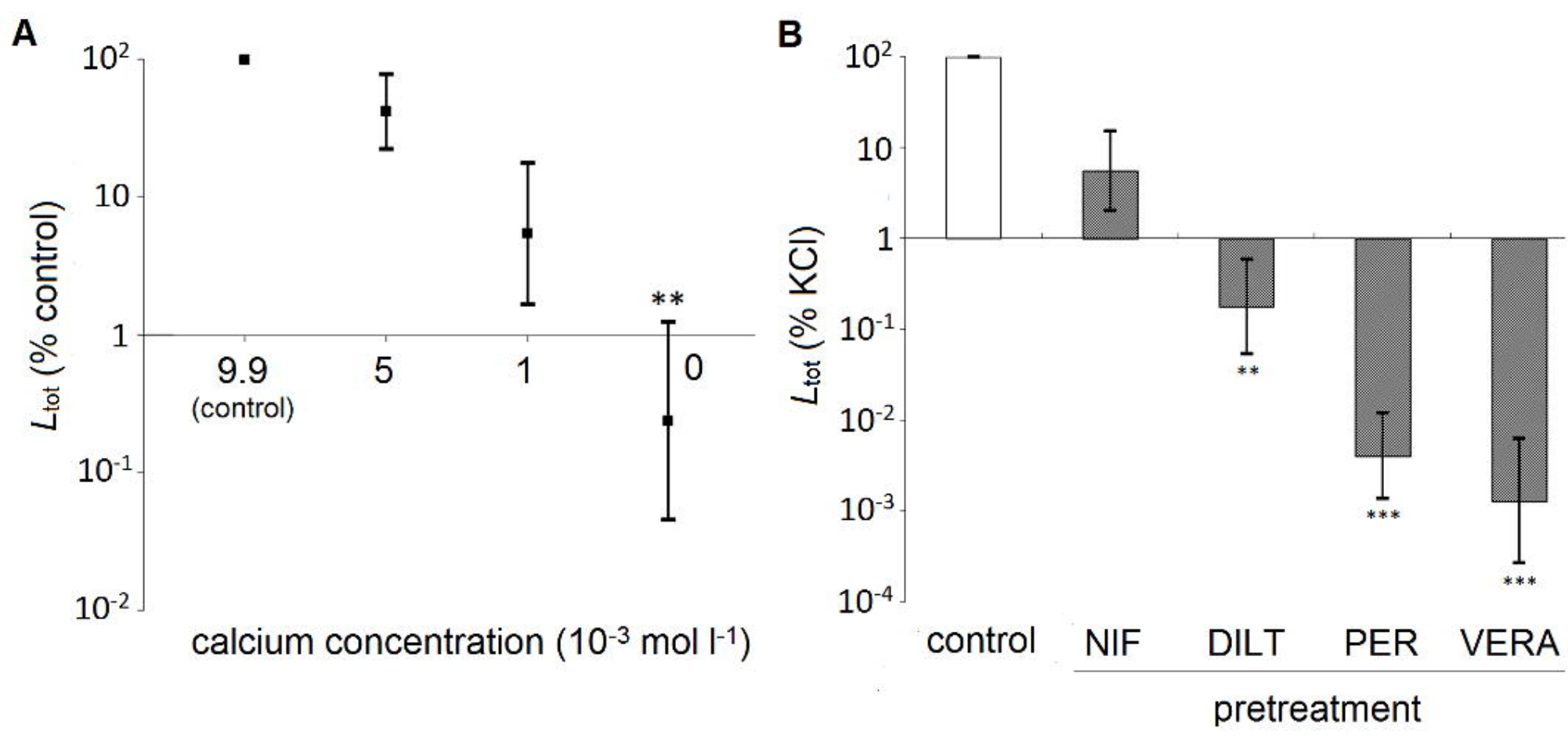
drug			concentration
usual name	commercial name	pharmacology	(mmol l^{-1})
KCl	potassium chloride	depolarizing agent	200
serotonin	5-hydroxytryptamine	serotoninergic neurotransmitter	1
epinephrine	(±)-epinephrine	adrenergic neurotransmitter	1
GABA	gamma-aminobutyric acid	GABAergic neurotransmitter and neuromodulator	1
nitric oxide	sodium nitroprusside	guanylyl cyclase activator	1
carbachol	carbamoylcholine chloride	cholinergic agonist	1
DMPP	1,1-dimethyl-4-phenyl-piperazinium iodide acetylcholine	nicotinic agonist	1
acetylcholine	chloride	cholinergic neurotransmitter	1
eserine	eserine	cholinesterase inhibitor	1
tubocurarine	d-tubocurarine chloride	cholinergic nicotinic receptor antagonist	1
atropine	atropine sulfate	cholinergic muscarinic receptor antagonist	1
nifedipine	nifedipine	calcium channel blocker	1
diltiazem	diltiazem chloride	calcium channel blocker	1
trifluoperazine	trifluoperazine dihydrochloride	calmodulin inhibitor	1
verapamil	(±)-verapamil	calcium channel blocker	1

drug	L_{tot} (% KCl)	p-value	n
ASW (control)	4.67 ± 4.22		5
serotonin	16.56 ± 15.13	1.000	5
epinephrine	53.71 ± 52.76	1.000	5
GABA	15.76 ± 10.60	1.000	5
nitric oxide	89.40 ± 79.55	1.000	4
carbachol	1129.88 ± 482.90	0,2839	5


A
20 Mq
30 s
C
10^5
10^4
10^3
10^2
10
1
L_{tot} (Mq)
KCl
carbachol
1
10
10^2
10^3
10^4
L_{max} (Mq s^{-1})
B


A
0.5 RLU
1 s
500 RLU
1 s
L_{tot} (% KCl)
10^3
10^2
10
1
10^{-1}
-6
-5
-4
-3
carbachol concentration (10^x mol l^{-1})
B
10^4
10^3
10^2
10
1
L_{tot} (% control)
control
eserine pretreatment
**
carbachol
acetylcholine
C
10^3
10^2
10
1
L_{tot} (% carbachol)
**
without pretreatment
tubocurarine
atropine
pretreatment


A
10^2
10
1
10^{-1}
10^{-2}
L_{tot} (% control)
9.9
(control)
5
1
0
**
calcium concentration (10^{-3} mol l^{-1})
B
10^2
10
1
10^{-1}
10^{-2}
10^{-3}
10^{-4}
L_{tot} (% KCl)
**
***
***
control
NIF
DILT
PER
VERA
pretreatment