ORIGINAL ARTICLE

GABA- and NO-ergic modulators control antinociceptive responses

Ivan Tyurenkov [1], Valentina Perfilova [1], Olga Vasil'eva [2], Ilya Rogachevskii [3], Valentina Penniyaynen [3], Tatiana Shelykh [3], Svetlana Podzorova [3], Boris Krylov [3], Vera Plakhova [3]

[1] Volgograd State Medical University, Volgograd, Russia; [2] Herzen State Pedagogical University of Russia, St. Petersburg, Russia; [3] Pavlov Institute of Physiology of the Russian Academy of Sciences, St. Petersburg, Russia.

Correspondence to: Prof. Boris V. Krylov, DSc., PhD., I.P. Pavlov Institute of Physiology, Russian Academy of Sciences, Laboratory of Physiology of Excitable Membranes, Nab. Makarova, 6 199034 St. Petersburg, Russian Federation. tel: +7(812)3281301; fax: +7(812)3280501; e-mail: krylov@infran.ru; krylovbv@yandex.ru

Submitted: 2018-02-28 Accepted: 2018-04-30 Published online: 2018-06-30

Key words: **NaV1.8 channels; nociceptive neurons; patch-clamp method; organotypic tissue culture; hot plate test; tail flick test; Randall-Selitto test; NO- and GABA-ergic systems; analgesic effect**

Act Nerv Super Rediviva 2018; **60**(1): 1–8 ANSR600118A01

Abstract

OBJECTIVES: Both NO-ergic and GABA-ergic systems are known to modulate the nociceptive system. However, there is no data available describing the physiological role of slow sodium channels ($Na_V1.8$) in antinociceptive response evoked by NO- and GABA-ergic system modulators. Our goal was to examine the central and peripheral analgesic effects possibly resulting from activation of NO-ergic and GABA-ergic signaling cascades in neurons.

METHODS: Effects of two-component agent RSPU-260 comprised of methyl-4-amino-3-phenylbutanoate hydrochloride and L-arginine hydrochloride was investigated by patch-clamp, organotypic nerve tissue culture and behavioral (hot plate, tail flick, Randall-Selitto) techniques.

RESULTS: Patch-clamp data indicate that only GABA-ergic system modulator, methyl-4-amino-3-phenylbutanoate hydrochloride, can control the effective charge transfer in the activation gating system of $Na_V1.8$ channels, inducing a weak effect at the spinal level. *In vivo* experiments demonstrate that both modulators (L-arginine and methyl-4-amino-3-phenylbutanoate hydrochloride) applied simultaneously at relatively low concentrations produce an effective analgesia at the spinal and supraspinal levels.

CONCLUSIONS: It is demonstrated that RSPU-260 evokes significant analgesic effects at both spinal and supraspinal levels. GABA-ergic modulator, methyl-4-amino-3-phenylbutanoate hydrochloride, controls the effective charge transfer in the activation gating system of $Na_V1.8$ channels responsible for nociceptive information coding. The second component of RSPU-260, L-arginine, is totally ineffective in respect to this target. A pronounced synergic effect at the spinal level can be achieved on the secondary sensory neuron in the dorsal horn whose synaptic membrane may be under NO-ergic system control. It is the unit where the nociceptive system can be additionally effectively regulated by NO- and GABA-ergic systems. At the supraspinal level, both NO- and GABA-ergic modulators activate their corresponding intracellular signaling cascades, thus resulting in a strong RSPU-260 analgesic effect.

Introduction

Voltage-dependent sodium channels ($Na_V1.1$ – $Na_V1.9$) are critically important molecular structures which provide and control the excitability of the nerve tissue, and it is of major interest to study their role in primary coding of nociceptive information. The initial unit of the nociceptive system is pain receptors (nociceptors) located virtually in all body tissues. Signal coding in nociceptive neurons is predominantly performed by slow tetrodotoxin-resistant sodium ($Na_V1.8$) channels (Kostyuk *et al* 1981; Gold *et al* 1996), functional activity of which is enhanced by mechanical damage, inflammatory processes or action of hyperalgesic agents (Akopian *et al* 1996; Gold *et al* 1996; Lai *et al* 2004), thus resulting in an increase in their impulse firing leading to a pronociceptive reaction at the organismal level.

On the contrary, a decrease in $Na_V1.8$ channel firing frequency should lead to an antinociceptive effect. Agents capable of reducing the excitability of nociceptors due to decreasing the voltage sensitivity of slow sodium channels are eligible for the role of analgesics (Krylov *et al* 2000; Krylov *et al* 2017). This specific modulation of $Na_V1.8$ channels allows the polymodal nociceptors to retain the ability to transmit signals of other modalities (for instance, tactile and temperature) by selectively turning off the relatively higher-frequency component of their impulse activity, which encodes the pain signal. It is therefore extremely promising to search for agents that are structurally similar to endogenous substances which safely and specifically modulate functional activity of slow sodium channels, as such agents may be utilized for development of novel analgesics. In this respect, it is very challenging to investigate the mechanisms of NO-ergic and GABA-ergic modulation of $Na_V1.8$ channels responsible for nociceptive signal coding.

Both NO-ergic and GABA-ergic systems are known to modulate the nociceptive system (Malan *et al* 2009; Meisner *et al* 2010; Cury *et al* 2011; Romero *et al* 2011; Romero *et al* 2012; Kukushkin & Igonkina 2014). However, there is no data available on involvement of slow sodium channels in NO-triggered cascade signaling. The effect of GABA-ergic cascade molecules on the activation gates of $Na_V1.8$ slow sodium channels is also poorly studied. To fill this gap, we have applied a two-component composition RSPU-260 comprised of methyl-4-amino-3-phenylbutanoate hydrochloride (Figure 1, A) and L-arginine hydrochloride (Figure 1, B), the former of which activates the GABA-ergic system (Enna & McCarson 2006; Martins *et al* 2015), while the latter modulates the NO-ergic system (Vivancos *et al* 2013; Talarek *et al* 2017; Safaripour *et al* 2018).

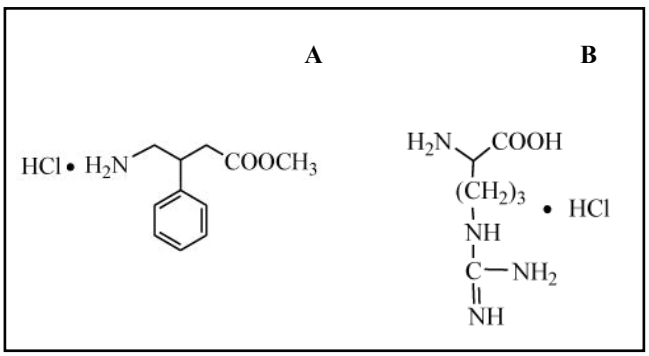

Fig. 1. Chemical structure of RSPU-260 components. **A**. methyl-4-amino-3-phenylbutanoate hydrochloride. **B**. L-arginine hydrochloride.

Methods

The experiments were designed in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). The Local Committees for Animal Care and Use at I.P. Pavlov Institute of Physiology and Volgograd State Medical University approved all experimental procedures with the animals. The animals were obtained from Rappolovo vivarium (Leningrad district, Russia) and Biocollection of I.P. Pavlov Institute of Physiology supported by Russian Federal Agency of Research Organizations.

Patch-clamp technique

Rat nociceptive neuron $Na_V1.8$ channels were investigated using the whole-cell patch-clamp method. Experiments were performed on short-term cultured dorsal root ganglia (DRG) neurons isolated from newborn *Wistar* rats. These nociceptive cells are characterized with high density of $Na_V1.8$ channels (Djouhri *et al* 2003). DRG were isolated from the L5-S1 region of the spinal cord and were placed in Hanks' solution. Enzymatic treatment (Kostyuk *et al* 1975), ion current recordings and data processing were performed as described earlier (Krylov *et al* 2000; Krylov *et al* 2017). The series resistance (*R*s) was constantly monitored in all the experiments and maintained below 3 MΩ (Osipchuk & Timin 1984). Reagents were from Sigma.

Organotypic tissue culture

In the following series of experiments, RSPU-260 effect on sensory ganglia neurites growth was investigated using organotypic embryonic nerve tissue culture as described in detail previously (Lopatina *et al* 2012). Experiments were performed on 10–12-day old chick embryo explants cultured on collagen support in Petri dishes at 36.5 °C and 5% CO_2 in CO_2 incubator (Sanyo, Japan) for three days. The nutrient medium contained 45% Hanks' solution and 40% Eagle's medium supplemented with insulin (0.5 units/ml), glucose (0.6%), glutamine (2 mM), gentamicin (100 units/ml), 5% chick embryo extract, and 10% bovine fetal serum. Explants cultured without exposure to the test substance served as the control. RSPU-260 was added to the culturing medium. Neurite outgrowth was evaluated quantita-

tively by the morphometric method. The area index (AI) was calculated as the ratio of the explant growth area to the area of the central zone of a ganglion. The AI value of the control explants was taken for 100%. Explants were visualized by Axio Observer Z1 microscope (Carl Zeiss, Germany) and further analyzed with ImageJ and ZEN_2012 software. Experiments were conducted using the equipment of the Confocal Microscopy Collective Use Center (I.P. Pavlov Institute of Physiology of the Russian Academy of Sciences).

Behavioral tests

Adult (three months old) male (n=12) and female (n=12) *Wistar* rats (170–190 g) were used in the study. The following groups of animals were formed, each including 4 males and 4 females. Two experimental groups of rats were intraperitoneally injected with RSPU-260 (12.5 mg/kg and 25 mg/kg) 30 minutes prior to the experiments in a volume of 0.1 ml/100 g of animal weight. In the intact group the animals were injected with physiological solution of the same volume.

Hot plate test. Evaluation of thermal somatic pain sensitivity was performed using "Hot/Cold Plate" device (Ugo Basile, Italy). The hot plate test is characterized by the most complicated organization of the behavioral reflex, and this technique makes it possible to evaluate the effect of the investigated substances on the nociceptive system at the supraspinal level (Kitchen & Crowder 1985). The animal was placed 30 minutes after the injection on a copper plate (the bottom of a plastic cylinder 15 cm in diameter) at a predetermined temperature (55 °C), and the latent time of appearance of nociceptive reaction (licking of hind paws) was recorded.

Tail flick test. «Tail flick analgesia-meter» (Ugo Basile, Italy) device was used to register the reaction time. Basal reaction time of animals to radiant heat was recorded by placing the tip (last 1–2 cm) of the tail on the radiant heat source. The tail withdrawal from the radiant heat source was taken as the end point. The test is based on the spinal flexor reflex, behavioral tail-tapping reaction upon exposure to infrared irradiation of the skin surface. In this test, C and Aδ fibers of the nociceptors are sequentially activated, including polymodal nociceptors and high-threshold mechanoreceptors. The latent period (LP) of tail withdrawal reaction was recorded (Bianchi & Franceschini 1954).

Randall-Selitto test. Evaluation of mechanical somatic pain resulting from formalin (a noxious pain-producing agent) injection was carried out by the Pressure Application Measurement (PAM) system (Ugo Basile, Italy) used to measure mechanical sensitivity in animal paws. To study the effect of substances on acute inflammation, the mechanical stimulation method was used on the background of formalin hyperalgesia (Randall-Selitto test). The basis of this test is the increase in afferent nociceptive firing produced by gradually increasing pressure against the backdrop of acute inflammation caused by a chemical stimulus. Mechanical hyperalgesia was induced by injecting 50 μl of 2% formalin solution into the ventrolateral surface of the paw subcutaneously, followed by pointwise application of a dosed incremental mechanical pressure to the injection site. The threshold (the weight in grams at which the reflex of paw withdrawal appeared) and LP (latent period, the time after the onset of compression, through which the reflex of paw withdrawal appeared) were measured. This test allows to evaluate both acute nociception (the first phase, 5–10 min after formalin injection) and tonic persistent nociception (the second phase, duration about 30–40 min). Specific pain-related paw withdrawal patterns in response to chemical and mechanical stimuli are organized at the spinal level (Randall & Selitto 1957; Dubuisson & Dennis 1977; Grechko *et al* 2016).

Statistical analysis

The data were processed with Student's *t* test. A *p*-value of ≤0.05 was considered to be statistically significant. To evaluate the differences between the independent samples in behavioral tests, the Kruskal-Wallis test with the Dunn's post-test was used.

Results

Patch-clamp data

Extracellular application of RSPU-260 (10 μM) resulted in a decrease of the voltage sensitivity of $Na_V1.8$ channels due to changes in the effective charge transfer (Z_{eff}) of their activation gating system. The limiting-slope procedure (Almers 1978) was used to estimate Z_{eff}. This approach has been described in our prior publications (Krylov *et al* 2000; Yachnev *et al* 2012; Krylov *et al* 2017). The ratio of the number of open channels (N_o) to the number of closed channels (N_c) is calculated as

$$N_o/N_c = G_{Na}(E)/[G_{Na}^{max} - G_{Na}(E)],$$

where G_{Na}^{max} and $G_{Na}(E)$ are the maximal value and the voltage dependence of the chord conductance, respectively. $G_{Na}(E)$ can be obtained in the patch-clamp experiments as

$$G_{Na}(E) = I_{max}(E)/(E - E_{Na}),$$

where I_{max} is the amplitude value of the sodium current, E_{Na} is the reversal potential for sodium ions. $G_{Na}(E)$ is a monotonous function which approaches its maximum value G_{Na}^{max} at positive potentials E. According to the Almers' theory, the limiting-slope procedure can be applied:

$$\lim_{E\to-\infty}(N_o/N_c) = \lim_{E\to-\infty}\{G_{Na}(E)/[G_{Na}^{max} - G_{Na}(E)]\} \to C\cdot\exp[(Z_{eff}e_0E)/(kT)], \quad E\to-\infty \text{ (Equation 1)}$$

where N_o is the number of open channels, N_c is the number of closed channels when the membrane potential E approaches minus infinity ($E \rightarrow -\infty$). The slope of the asymptote passing through the first points determined by the most negative values of E makes it possible to estimate Z_{eff} (Equation 1), since the Boltzmann's principle is applicable at these potentials, where k is the Boltzmann constant, T is the absolute temperature, C is a constant, e_0 is the electron charge.

The families of $Na_V1.8$ channel currents in the control experiment and after extracellular application of RSPU-260 are presented in Figure 2, A. It is clearly seen that the decaying phase of sodium current is accelerated pronouncedly. The corresponding peak current-voltage curve shifts slightly to the left after RSPU-260 application (Figure 2, B). The left branch of this function is more flat than in the control experiment. The voltage dependencies of the chord conductance are also different at the very negative E between the control and RSPU-260 data (Figure 3, A). When the chord conductance dependencies are obtained, the Almers' limiting-slope procedure (Equation 1) can be applied, making it possible to evaluate Z_{eff} by constructing the voltage dependence of the logarithmic voltage sensitivity function $L(E)$:

$$L(E) = ln\,(G_{Na}(E)/(G_{Na}^{max} - G_{Na}(E)) \qquad \text{(Equation 2)}$$

The asymptote passing through the first points of $L(E)$ function obtained at the most negative values of the membrane potential E allows to calculate the Z_{eff}

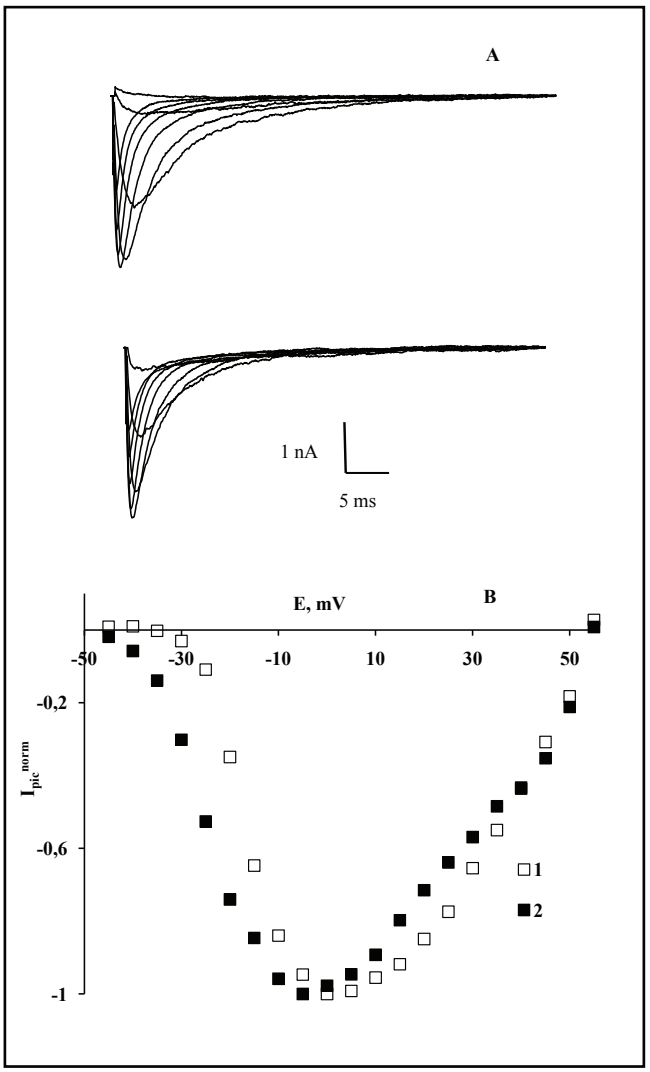

Fig. 2. Effects of RSPU-260 on $Na_V1.8$ channels. **A**. Families of sodium currents measured in the control experiment (top) and after application of RSPU-260 at 10 μM (bottom). The test potential was changed from –35 mV to 35 mV with a step of 10 mV. The holding potential of 500-ms duration was equal to –110 mV in all records. The leakage and capacitive currents were subtracted automatically. **B**. Negative shift of the normalized peak current-voltage curve after application of RSPU-260. 1 – control data; 2 – after application of RSPU-260.

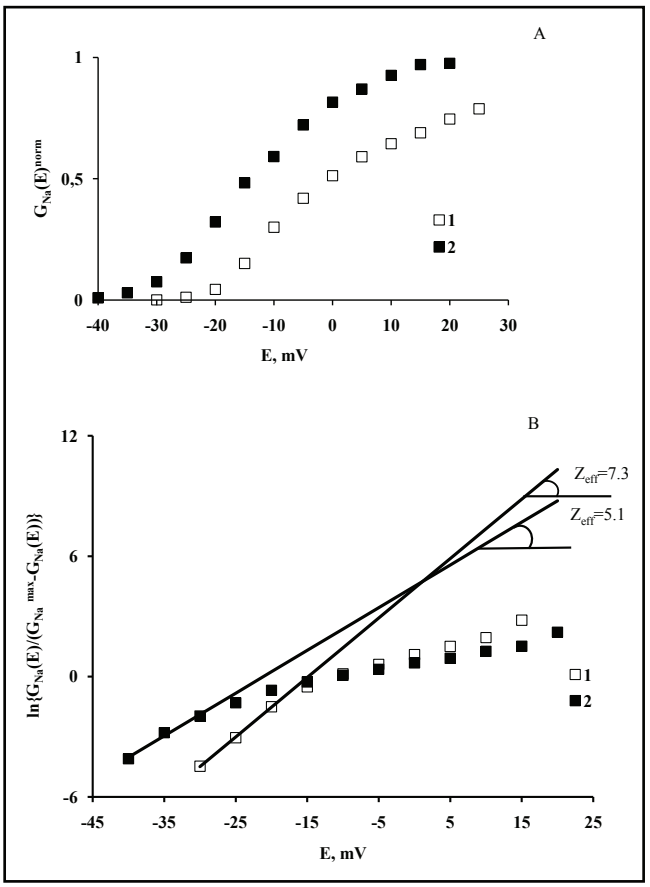

Fig. 3. Decrease of the effective charge of $Na_V1.8$ channel activation gating device after application of RSPU-260. **A**. Voltage dependence of the normalized peak conductance used for Z_{eff} evaluation. The function $G_{Na}(E)$ was normalized, i.e., we plotted $G_{Na}(E)^{norm} = G_{Na}(E)/\ G_{Na}^{max}(E)$, where $G_{Na}^{max}(E)$ was the maximal value of $G_{Na}(E)$. The results of the control experiments and after application of RSPU-260 at 10 μM are presented. 1 – control data; 2 – after application of RGPU-260; **B**. Z_{eff} evaluation by the Almers' limiting-slope procedure in the control experiment and after application of RSPU-260 at 10 μM. The exponential function presented in the logarithmic scale (the axis of ordinates) makes it possible to evaluate Z_{eff} from the slopes of the asymptotes passing through the first points determined by the most negative values of the membrane potential in the control experiments and after application of RSPU-260 at 10 μM. 1 – control data; 2 – after application of RSPU-260.

value, which is linearly proportional to the tangent of the asymptote slope (Figure 3, B). Our results show that application of RSPU-260 (10 μM) results in a significant decrease of Z_{eff} from 6.8±0.4 (n=21) to 5.0±0.3 (n=19) (Figure 4). These data indicate that RSPU-260 is able to modulate slow sodium channels ($Na_V1.8$), inducing a relatively weak effect on primary sensory neuron.

Organotypic tissue culture

During the first day of embryonic nerve tissue culturing, the explants spread over the collagen substrate; neurite growth and eviction of proliferating and migrating cells begins. After three days of culturing, two distinct zones can be recognized both in the control and experimental sensory ganglia explants. The central zone is composed of nonmigrating differentiating neuroblasts and the peripheral zone (also termed the growth zone) consists of fibroblast-like cells, glia and growing neurites; synaptic connections are not yet formed (Figure 5). RSPU-260 effect was investigated using the area index (AI), an extremely sensitive parameter to examine the exposure of nerve cells to diverse substances. The agent demonstrated no influence on embryonic nerve tissue growth in the range of concentrations from 0.01 nM to 100 μM, as the difference between the experimental and control AI values was not statistically significant (Figure 6).

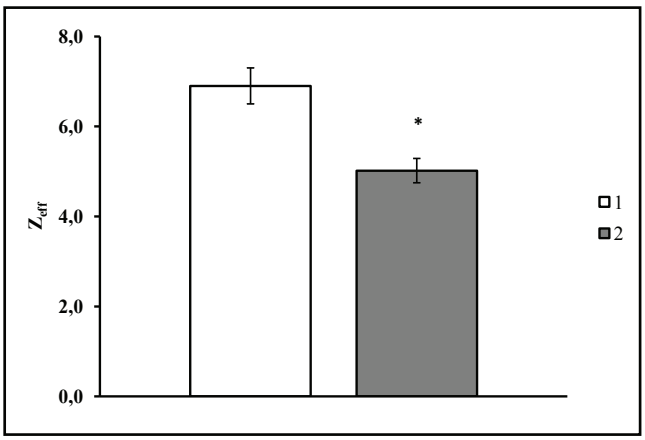

Fig. 4. Influence of RSPU-260 on the effective charge of $Na_V1.8$ channel activation gating system. The control value of Z_{eff} = 6.8±0.4 (n=21). Z_{eff} is reduced to 5.0±0.3 (n=19) after application of RSPU-260 at 10 μM. * - difference between experimental and control data is statistically significant. 1 – control data; 2 – after application of RSPU-260.

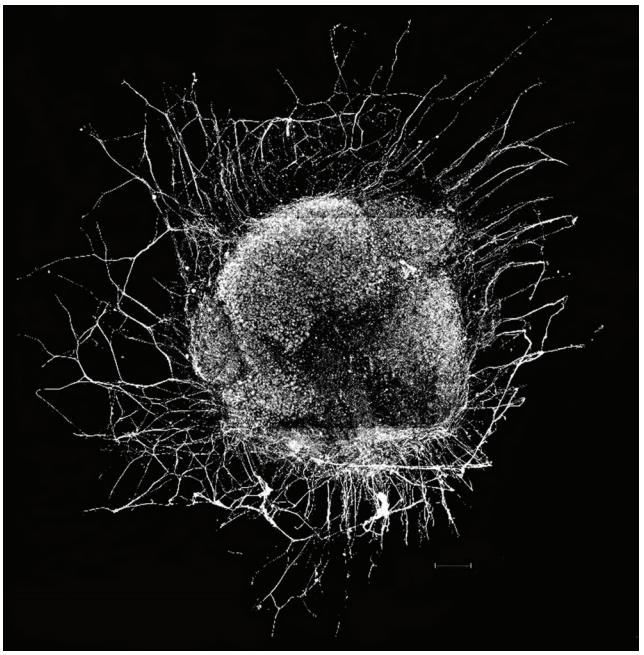

Fig. 5. Microphotograph of a sensory ganglion of 10-day old chick embryo. Control data, the nutrient medium, third day of culturing. The ganglion is stained with anti-neurofilament antibody (Carl Zeiss). Scale bar 200 μm.

Behavioral tests

Hot plate test. The hot plate test results for RSPU-260 applied at two concentrations revealed a clear tendency for the latent period (LP) of hind paw licking to increase with increasing the agent concentration (Figure 7, A). A statistically significant analgesic effect was observed at 25 mg/kg. In this case, LP is 14.9±2.9 s, 49% (p<0.05) higher than in the control (intact) group with LP of 10.0±1.7 s (Figure 7, A), which indicates that RSPU-260 (25 mg/kg) modulates the nociceptive response at the supraspinal level.

Tail flick test. Figure 7, B illustrates the results obtained with the tail flick test when exposed to two concentrations of RSPU-260 (12.5 and 25 mg/kg). An increase in the agent concentration led to an increase in the behavioral response, but the observed effect was statistically insignificant. The tail flick test, which evaluates the purely spinal withdrawal reflex, indicates a very weak effect of RSPU-260 on the peripheral nociceptive system.

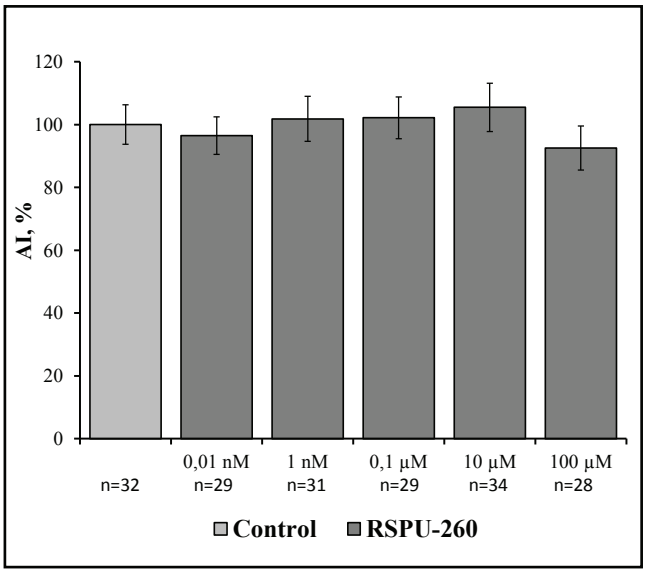

Fig. 6. RSPU-260 effect on neurite growth of sensory ganglion explants of 10–12-day old chick embryos.

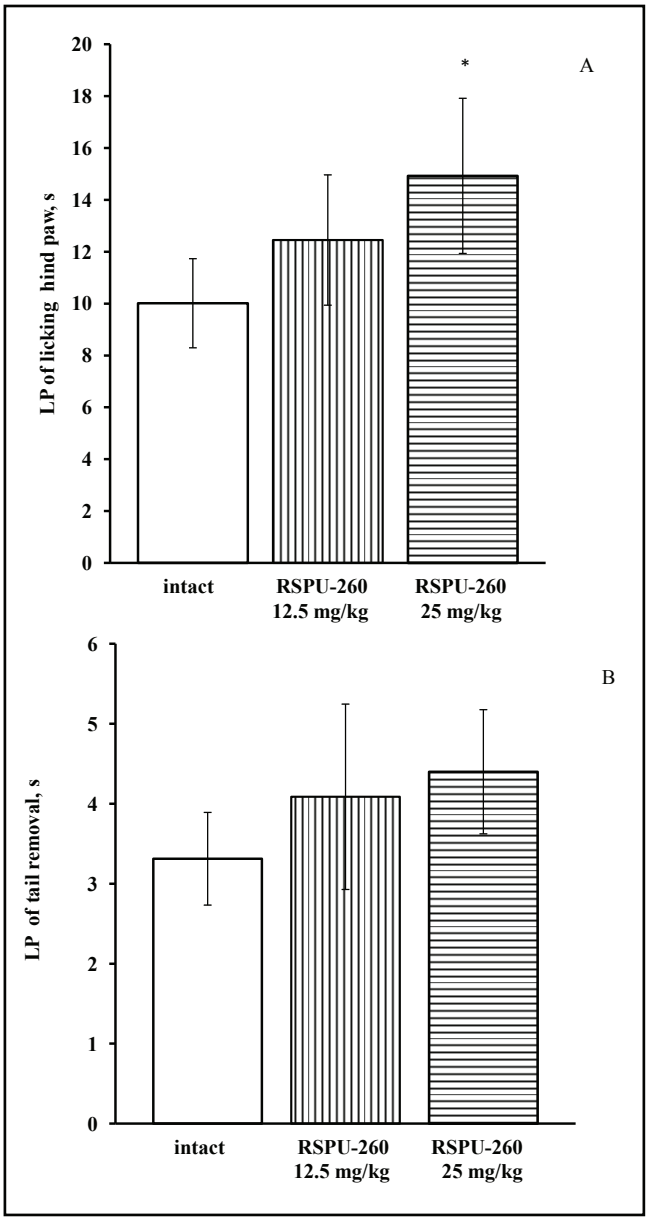

Fig. 7. Effects of RSPU-260 on antinociceptive behavioral reactions in the hot plate and tail flick tests. **A** – Hot plate test. RSPU effect on latent period (LP) of hind paw licking; **B** – Tail flick test. RSPU-260 effect on latent period of tail removal.
*– difference between experimental and control data is statistically significant.

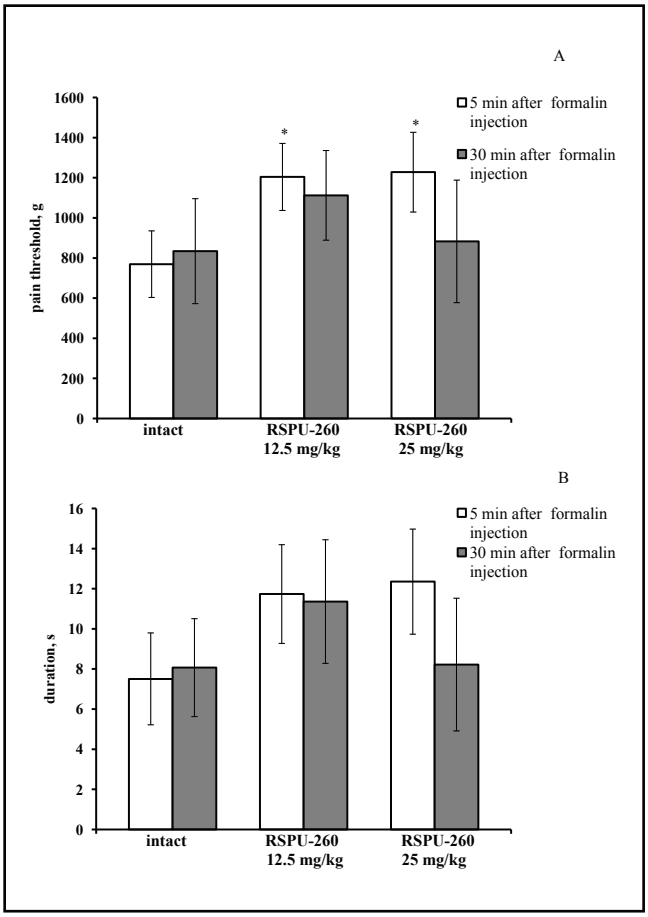

Fig. 8. Effects of RSPU-260 on antinociceptive behavioral reactions in the Randall-Selitto test. **A** – RSPU-260 effect on pain threshold of hind paw removal under mechanical stimulation; **B** – RSPU-260 effect on latent period (LP) of hind paw removal. *– difference between experimental and control data is statistically significant.

Randall-Selitto test. The Randall-Selitto test revealed that RSPU-260 at both applied concentrations statistically significantly increased the acute pain threshold of the paw withdrawal response (1204±167 and 1228±198 g, respectively) as compared to the control group of animals (769±165 g) by 56 and 60% (Figure 8, A). However, only a tendency to develop an antinociceptive reaction to acute pain was observed considering LP as the parameter (Figure 8, B). No significant effect of RSPU-260 on persistent inflammatory pain was detected (Figure 8, A, B).

Thus, the data obtained testify to the expressed antinociceptive action of the investigated agent: RGPU-260 was demonstrated to suppress the nociceptive response also at the spinal level.

Discussion

When developing new drugs, the most promising approach is to use endogenous substances or structurally similar molecules which can effectively and safely correct pathological conditions on the organismal level. RSPU-260 meets these criteria: it is comprised of methyl-4-amino-3-phenylbutanoate hydrochloride and L-arginine hydrochloride, the former of which activates the GABA-ergic system, while the latter modulates the NO-ergic system.

Such an approach has already made it possible to successfully apply for clinical purposes in Russia a number of drugs that modulate these systems (phenibut, phenotropil, picamilonum, etc.). R&D projects based on similar ideas allowed to obtain several medicinal substances with high neurotropic and psychotropic

activity, as well as with positive cardiovascular action (Tyurenkov *et al* 2012a, b; Lapin 2001). Simultaneous modulation of both GABA- and NO-ergic systems by RSPU-260 is expected to have a synergic effect resulting in pain relief.

Finding of a strong analgesic effect exhibited by RSPU-260 (25 mg/kg) at the supraspinal level is the main result of the present work demonstrated *in vivo* in the hot plate test. In the tail flick test, the same concentration of the agent produced a very weak response, indicating only a tendency to display an antinociceptive action at the spinal level. The significant spinal antinociceptive effect of RSPU-260 was detected only with the help of more sophisticated Randall-Selitto test.

Our patch-clamp experiments registered, however, just a moderate RGPU-260 antinociceptive effect. The mechanisms that could account for the data obtained may be as follows. L-arginine does not affect the activation gating device of $Na_V1.8$ channels (Plakhova *et al* 2016), which means that another RSPU-260 component, a GABA-ergic modulator methyl-4-amino-3-phenylbutanoate hydrochloride, is responsible for the decrease in the voltage sensitivity of these channels. This modulator of the GABA-ergic system is for the first time demonstrated here to act upon another target ($Na_V1.8$ channels), thus providing an antinociceptive influence on primary sensory neuron. The effect of this agent (10 μM) is nevertheless relatively weak as compared to that of comenic acid (100 nM), a potent and specific modulator of $Na_V1.8$ channels (Plakhova *et al* 2014). On the other hand, it should be emphasized that a comparably potent analgesic effect is observed at almost the same doses of the agents applied *in vivo*: RSPU-260, 25 mg/kg; comenic acid, 30 mg/kg (Plakhova *et al* 2014).

RSPU-260 was shown to be ineffective in organotypic embryonic nerve tissue culture; the agent does not control the growth of sensory ganglia neurites. This means that all *in vitro* experiments (patch-clamp and tissue culture) were performed on primary sensory neurons that lack synaptic connections.

It is well known that the NO-ergic system controls signaling mechanisms across the synaptic cleft (Cury *et al* 2011). Therefore, it is not surprising that a modulator of the NO-ergic system (L-arginine) did not produce any effect at the level of primary sensory neuron *in vitro* (Plakhova *et al* 2016). However, interaction between the NO-ergic system and the nociceptive system becomes possible after the first signal switching to secondary sensory neuron in the dorsal horns, which should be manifested at the spinal level due to the fact that L-arginine can affect this neuron across the synaptic cleft. At the supraspinal level, where the mechanisms of synaptic transmission play a central role, both NO- and GABA-ergic modulators find their targets on synaptic membranes, which results in a strong analgesic effect.

Conclusions

Investigated two-component agent RSPU-260 is comprised of NO- and GABA-ergic system modulators, and it was demonstrated *in vivo* to evoke significant analgesic effects at both spinal and supraspinal levels when applied at relatively low concentration (25 mg/kg). Our data obtained *in vitro* indicate that only methyl-4-amino-3-phenylbutanoate hydrochloride, a GABA-ergic modulator, can control the effective charge transfer in the activation gating system of $Na_V1.8$ channels responsible for nociceptive information coding. The second component of RSPU-260, L-arginine, is totally ineffective in respect to this target. A pronounced synergic effect at the spinal level can be achieved on secondary sensory neuron in the dorsal horn whose synaptic membrane may be under NO-ergic system control. It is the unit where the nociceptive system can be additionally effectively regulated by NO- and GABA-ergic systems. At the supraspinal level, both NO- and GABA-ergic modulators activate their corresponding intracellular signaling cascades, thus resulting in a strong analgesic effect.

Acknowledgement

The present work was funded by the Russian Foundation for Basic Research, project No. 18-015-00071.

REFERENCES

1 Akopian AN, Sivilotti L, Wood JN (1996). A tetrodotoxin-resistant voltage-gated sodium channel expressed by sensory neurons. *Nature*. **379**: 257–262.
2 Almers W (1978). Gating currents and charge movements in excitable membranes. *Rev Physiol Biochem Pharmacol.* **82**: 97–190.
3 Bianchi C & Franceschini J (1954). Experimental observations on Haffner's method for testing analgesic drugs. *Br J Pharmacol Chemother*. **9**: 280–284.
4 Cury Y, Picolo G, Gutierrez VP, Ferreira SH (2011). Pain and analgesia: The dual effect of nitric oxide in the nociceptive system. *Nitric Oxide*. **25**: 243–254.
5 Djouhri L, Fang X, Okuse K, Wood J, Berry C, Lawson S (2003). The TTX-resistant sodium channel $Na_V1.8$ (SNS/PN_3): expression and correlation with membrane properties in rat nociceptive primary afferent neurons. *J Physiol.* **550**: 739–752.
6 Dubuisson D & Dennis SG (1977). The formaline test: a quantitative study of the analgesic effects of morphine, meperedine and brain stem stimulation in rats and cats. *Pain*. **4**: 161–174.
7 Enna SJ & McCarson KE (2006). The role of GABA in the mediation and perception of pain. *Adv Pharmacol.* **54**:1–27.
8 Gold M, Reichling D, Shuster M, Levine J (1996). Hyperalgesic agents increase a tetrodotoxin-resistant Na current in nociceptors. *Proc Natl Acad Sci USA*. **93**: 1108–1112.
9 Grechko OYu, Eliseeva NV, Spasov AA, Litvinov RA (2016). Analgetic activity of a benzimidazole derivative on models of inflammatory pain (In Russian with English abstract). *J of Volgograd State Medical University*. **2**: 101–103.
10 Kitchen I & Crowder M (1985). Assessment of the hot-plate antinociceptive test in mice. A new method for the statistical treatment of graded data. *J Pharmacol Methods*. **13**: 1–7.

11 Kostyuk P, Krishtal O, Pidoplichko V (1975). Effect of internal fluoride and phosphate on membrane currents during intracellular dialysis of nerve cells. *Nature.* **257**: 691–693.
12 Kostyuk P, Veselovsky N, Tsyndrenko A (1981). Ionic currents in the somatic membrane of rat dorsal root ganglion neurons I – Sodium currents. *Neuroscience.* **6**: 2423–2430.
13 Krylov B, Derbenev A, Podzorova S, Lyudyno M, Kuz'min A, Izvarina N (2000). Morphine decreases the voltage sensitivity of slow sodium channels. *Neurosci Behav Physiol.* **30**: 431–39.
14 Krylov BV, Rogachevskii IV, Shelykh TN, Plakhova VB (2017). Frontiers in pain science. Volume 1. New non-opioid analgesics: understanding molecular mechanisms on the basis of patch-clamp and quantum-chemical studies. Sharjah: Bentham Science Publishers Ltd.
15 Kukushkin ML & Igonkina SI (2014). Significance of GABA in pain syndrome pathogenes (In Russian with English abstract). *Patol Fiziol Eksp Ter*. **1**: 68–78.
16 Lai J, Porreca F, Hunter JC, Gold MS (2004). Voltage-gated sodium channels and hyperalgesia. *Ann Rev Pharmacol Toxicol.* **44**: 371–397.
17 Lapin I (2001). History of drug development. Phenibut (β-Phenyl-GABA): a tranquilizer and nootropic drug. *CNS Drug Reviews.* **7**: 471–481.
18 Lopatina EV, Yachnev IL, Penniyaynen VA, Plakhova VB, Podzorova SA, Shelykh TN *et al* (2012). Modulation of signal-transducing function of neuronal membrane Na+,K+-ATPase by endogenous ouabain and low-power infrared radiation leads to pain relief. *Med Chem*. **8**: 33–39.
19 Malan TP, Mata HP, Porreca F (2002). Spinal GABA(A) and GABA(B) receptor pharmacology in a rat model of neuropathic pain. *Anesthesiology*. **96**: 1161–1167.
20 Martins I, Carvalho P, de Vries MG, Teixeira-Pinto A, Wilson SP, Westerink BH *et al* (2015). GABA acting on $GABA_B$ receptors located in a medullary pain facilitatory area enhances nociceptive behaviors evoked by intraplantar formalin injection. *Pain.* **156**:1555–1565.
21 Meisner JG, Marsh AD, Marsh DR (2010). Loss of GABAergic interneurons in laminae I-III of the spinal cord dorsal horn contributes to reduced GABAergic tone and neuropathic pain after spinal cord injury. *J Neurotrauma*. **27**: 729–737.
22 Osipchuk Y & Timin E (1984). Electrical measurements on professed cells. In: Kostyuk P & Kryishtal O, editors. Intracellular perfusion of excitable cells. London: Pergamon press. p. 103–129.
23 Plakhova V, Rogachevsky I, Lopatina E, Shelykh T, Butkevich I, Mikhailenko V, *et al* (2014). A novel mechanism of modulation of slow sodium channels: from ligand-receptor interaction to design of an analgesic medicine. *Act Nerv Super Rediviva* **56**: 55–64.
24 Plakhova VB, Rogachevsky IV, Shelykh TN, Podzorova SA, Krylov BV (2016). Cyclic polypeptide PP-14 modulates the voltage sensitivity of slow sodium channels (In Russian with English abstract). *Sensornye Systemy.* **30**: 234–240.
25 Randall LO & Selitto JJ (1957). A method for measurement of analgesic activity on inflamed tissue. *Arch Int Pharmacodyn Ther*. **111**: 409–419.
26 Romero TR, Galdino GS, Silva GC, Resende LC, Perez AC, Cortes SF *et al* (2012). Involvement of the L-arginine/nitric oxide/cyclic guanosine monophosphate pathway in peripheral antinociception induced by N-palmitoyl-ethanolamine in rats. *J Neurosci Res*. **90**: 1474–1479.
27 Romero TR, Resende LC, Duarte ID (2011) The neuronal NO synthase participation in the peripheral antinociception mechanism induced by several analgesic drugs. *Nitric Oxide*. **25**: 431–435.
28 Safaripour S, Nemati Y, Parvardeh S, Ghafghazi S, Fouladzadeh A, Moghimi M (2018). Role of L-arginine/SNAP/NO/cGMP/KATP channel signalling pathway in antinociceptive effect of α-terpineol in mice. *J Pharm Pharmacol*. [Epub ahead of print]
29 Talarek S, Listos J, Orzelska-Gorka J, Jakobczuk M, Kotlinska J, Biala G (2017). The importance of L-Arginine:NO:cGMP pathway in tolerance to flunitrazepam in mice. *Neurotox Res.* **31**: 309–316.
30 Tyurenkov IN, Borodkina LE, Bagmetova VV (2012a) Functional aspects of neuroprotective effects of new salts and compositions of baclofen in the convulsive syndrome caused by electroshock. *Bul Exp Biol Med*. **153**: 710–713.
31 Tyurenkov IN, Bagmetova VV, Borodkina LE, Berestovitskaya VM, Vasil'eva OS (2012b). Fenibut and its citrate prevent psychoneurological disorders caused by chronic stress (paradoxical sleep deprivation) (In Russian with English abstract). *Eksp Klin Farmakol*. **75**: 8–13.
32 Vivancos GG, Parada CA, Ferreira SH (2003). Opposite nociceptive effects of the arginine/NO/cGMP pathway stimulation in dermal and subcutaneous tissues. *Br J Pharmacol*. **138**: 1351–1357.
33 Yachnev I, Plakhova V, Podzorova S, Shelykh T, Rogachevsky I, Krylov B (2012). Mechanism of pain relief by low-power infrared irradiation: ATP is an IR-target molecule in nociceptive neurons. *Med Chem.* **8**: 14–21.