Type 1 and type 3 ryanodine receptors are selectively involved in muscarinic antinociception in mice: An antisense study.

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TYPE 1 AND TYPE 3 RYANODINE RECEPTORS ARE SELECTIVELY INVOLVED IN MUSCARINIC ANTINOCICEPTION IN MICE: AN ANTISENSE STUDY

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Abstract—The importance of an intracellular calcium content increase to obtain cholinergic antinociception was demonstrated. The physiological and pathological role of ryanodine receptors (RyRs), receptors involved in the mobilization of intracellular calcium stores, at the CNS level is poorly understood. The aim of the present study was, therefore, to investigate the role of supraspinal endoplasmic type 1, 2 and 3 RyR subtypes in muscarinic antinociception in conditions of acute thermal (hotplate test) and inflammatory (abdominal constriction test) pain. In the absence of isoform selective RyR antagonists, types 1, 2 and 3 RyR knockdown mice were obtained. Western blotting experiments were performed to quantify the RyR isoform protein levels in knockdown mice demonstrating a selective protein level reduction in knockdown animals. I.c.v. pretreatment with an antisense oligonucleotide (aODN) against type 1 or type 3 RyR prevented cholinergic antinociception in the hotplate test shifting to the right of the physostigmine dose-response curve. This antagonistic effect disappeared 7 days after the end of the aODN administration. Conversely, the physostigmine analgesia remained unmodified in type 2 RyR knockdown mice. Similar results were obtained in the abdominal constriction test. Mice undergoing aODN treatments showed neither alteration of animals' gross behavior nor locomotor impairment (rota-rod and hole board tests). These results elucidate the intracellular mechanism underlying muscarinic antinociception. A selective involvement of RyR1 and RyR3 in supraspinal muscarinic analgesia was demonstrated whereas RyR2 appears not to play an essential role in acute thermal and inflammatory pain. © 2008 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: muscarinic receptor, analgesia, type 1 ryanodine receptors, type 2 ryanodine receptors, type 3 ryanodine receptors, intracellular Ca^{2+}.

The critical involvement of the cholinergic system in pain inhibitory pathways has long been known. Several studies aimed to elucidate the muscarinic receptor subtype in-

volved in the modulation of pain perception reported that supraspinal cholinergic antinociception is mediated by M_1 receptors (Bartolini et al., 1992; Iwamoto and Marion, 1993; Ghelardini et al., 1996, 2000; Naguib and Yaksh 1997). Post-receptorial events involved in muscarinic antinociception have also been recently investigated. M_1 receptors typically couple via the α subunits of the $G_{q/11}$ family to activate phospholipase C (PLC), stimulating phosphoinositide (PI) hydrolysis (Caulfield and Birdsall, 1998). Receptor-mediated activation of PLC results in the generation of at least two messengers, inositol-1,4,5-triphosphate ($InsP_3$) and diacylglycerol (DAG). The main effect of DAG is to activate protein kinase C (PKC); the effect of $InsP_3$ is to release Ca^{2+} stored in the endoplasmic reticulum (Caulfield and Birdsall, 1998). The importance of the receptor-mediated increase of the intracellular calcium content to obtain an antinociceptive effect by administration of cholinomimetics was recently evidenced. The inhibition of the IP_3 synthesis by pretreatment with LiCl and the blockade of IP_3 receptors (IP_3R) by administration the IP_3R antagonist heparin dose dependently prevented physostigmine and oxotremorine antinociception (Galeotti et al., 2003).

The concentration of intracellular Ca^{2+} is regulated by various mechanisms related to physiological functions. One mechanism is the influx of Ca^{2+} via Ca^{2+} channels through the plasma membrane. Another is the release of Ca^{2+} from intracellular stores via intracellular Ca^{2+}-release channels, the inositol 1,4,5-trisphosphate receptor ($InsP_3R$) and the ryanodine receptor (RyR): $InsP_3R$ is a key molecule for $InsP_3$-induced Ca^{2+} release, whereas RyR is important for Ca^{2+}-induced Ca^{2+} release (Mikoshiba, 1997; Fill and Copello, 2002).

Three types of RyR (RyR1-3) have been described; each is the product of a different gene. The products of the three RyR genes in mammals are named according to the tissue in which they were first identified. The skeletal RyR (RyR1) is the major isoform expressed in skeletal muscle and in cerebellar Purkinje cells. The cardiac RyR (RyR2) is the major isoform in myocardium and brain. The brain isoform (RyR3), although expressed in the CNS, is more common in other cell types including smooth muscle and lymphocytes (Fill and Copello, 2002). The RyR channels, containing four approximately 560 kDa subunits, are the largest known ion channel proteins. The three mammalian RyR isoforms, with approximately 5000 amino acid residues per subunit, show strong sequence homology: 66% between RyR1 and RyR2; 67% between RyR1 and RyR3; and 70% between RyR2 and RyR3 (Dulhunty and Pouliquin, 2003). Although the best-known function of

RyRs is to provide the Ca^{2+} trigger for muscle contraction, the channels may also play important roles in diverse cell signaling pathways. These processes are poorly understood and, in particular, little is known about the role of RyRs at the CNS level.

Recently, the involvement of RyRs in muscarinic antinociception has been hypothesized since the increase of pain threshold induced by cholinomimetics is prevented by pretreatment with the RyR antagonist ryanodine (Galeotti et al., 2005). The alkaloid ryanodine binds with high affinity to the RyR proteins inducing a complex change in single RyR channel function (Sutko et al., 1997). This change is similar in all three channel isoforms. The aim of the present study was, therefore, to investigate the role of RyR1, -2, and -3 in the intracellular mechanism of muscarinic analgesia at a supraspinal level in experimental conditions of acute thermal and inflammatory pain. To this purpose we generated RyR1, RyR2 and RyR3 knockdown mice selectively inhibiting the expression of each RyR subtype by using antisense oligonucleotides (aODNs).

EXPERIMENTAL PROCEDURES

Animals

Male Swiss albino mice (24–26 g) from Morini (San Polo d'Enza, Italy) were used. Fifteen mice were housed per cage. The cages were placed in the experimental room 24 h before the test for acclimatization. The animals were fed a standard laboratory diet and tap water *ad libitum* and kept at 23 ± 1 °C with a 12-h light/dark cycle, light at 7 a.m. All experiments were carried out in accordance with the European Community Council's Directive of 24 November 1986 (86/609/EEC) relative to experimental animal care and conformed to local guidelines on the ethical use of animals. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Hotplate test

Mice were placed inside a stainless steel container, which was set thermostatically at 52.5 ± 0.1 °C in a precision waterbath from KW Mechanical Workshop, Siena, Italy. Reaction times (s), were measured with a stopwatch before and 15, 30, 45 and 60 min after administration of the analgesic drug. The endpoint used was the licking of the forepaws or hind paws. Those mice scoring less than 12 and more than 18 s in the pretest were rejected (30%). An arbitrary cutoff time of 45 s was adopted.

Abdominal constriction test

Mice were injected i.p. with a 0.6% solution of acetic acid (10 ml kg^{-1}). The number of stretching movements was counted for 10 min, starting 5 min after acetic acid injection. Physostigmine and ryanodine were administered 20 and 5 min before acetic acid injection, respectively.

Rota-rod test

The apparatus consisted of a base platform and a rotating rod with a diameter of 3 cm and a non-slippery surface. The rod was placed at a height of 15 cm from the base. The rod, 30 cm in length, was divided into five equal sections by six disks. Thus, up to five mice were tested simultaneously on the apparatus, with a rod-rotating speed of 16 r.p.m. The integrity of motor coordination was assessed on the basis of the number of falls from the rod in 30 s according to Vaught et al. (1985). Those mice scoring less

than three and more than six falls in the pretest were rejected (20%). The performance time was measured before (pretest) and 15, 30 and 45 min after the beginning of the test.

Hole-board test

The hole board test consisted of a 40 cm square plane with 16 flush mounted cylindrical holes (3 cm diameter) distributed four by four in an equidistant, grid-like manner. Mice were placed on the center of the board one by one and allowed to move about freely for a period of 10 min each. Two electric eyes, crossing the plane from mid-point to mid-point of opposite sides, thus dividing the plane into four equal quadrants, automatically signaled the movement of the animal (counts in 5 min) on the surface of the plane (spontaneous mobility). Miniature photoelectric cells, in each of the 16 holes, recorded (counts in 5 min) the exploration of the holes (exploratory activity) by the mice.

I.c.v. injection technique

I.c.v. administration was performed under ether anesthesia with isotonic saline as solvent. During anesthesia, mice were grasped firmly by the loose skin behind the head. A hypodermic needle (0.4 mm external diameter) attached to a 10 μl syringe was inserted perpendicularly through the skull and no more than 2 mm into the brain of the mouse, where 5 μl solution were then administered. The injection site was 1 mm to the right or left from the midpoint on a line drawn through to the anterior base of the ears. Injections were performed randomly into the right or left ventricle. To ascertain that solutions were administered exactly into the cerebral ventricle, some mice were injected with 5 μl of diluted 1:10 india ink and their brains were examined macroscopically after sectioning. The accuracy of the injection technique was evaluated with 95% of injections being correct.

Drugs

The following drugs were used: physostigmine hemisulphate; pyrilamine maleate, (±)baclofen (Sigma, Milan, Italy); ryanodine (Calbiochem, Milan, Italy); D-amphetamine, diphenhydramine hydrochloride (De Angeli, Rome, Italy). Other chemicals were of the highest quality commercially available. Drugs were dissolved in isotonic (NaCl 0.9%) saline solution immediately before use. Drug concentrations were prepared so that the necessary dose could be administered in a volume of 10 ml kg^{-1} by s.c. injection.

Doses and administration schedule for each compound were chosen on the bases of time-course and dose-response curves performed in our laboratory.

aODNs

Phosphodiester ODNs protected by terminal phosphorothioate double substitution (capped ODNs) against possible exonuclease-mediated degradation were purchased from Tib-Molbiol (Genoa, Italy). The sequences are the following: anti-RyR1: 5′-T*G*C CCC TGA CAT GTC C*C*C-3′; anti-RyR2: 5′-T*T*C GCC CGC ATC AGC C*A*T-3′; anti-RyR3: 5′-A*G*A TGC TAA TTG CAT C*T*C-3′ (* indicates the phosphorothioate residues). An 18-mer fully degenerated oligonucleotide (dODN), 5′-N*N*N NNN NNN NNN NNN N*N*N -3′ (where N is G, or C, or A, or T), was used as a control ODN. ODNs were vehiculated intracellularly by an artificial cationic lipid (DOTAP, Sigma) to enhance both uptake and stability. aODNs or dODNs were preincubated at 37 °C for 30 min with 13 μM DOTAP and supplied to mice by i.c.v. injection of a 5 μl solution on days 1, 2 and 3. Behavioral tests were performed on day 4, 18 h after the last i.c.v. injection of ODNs.

Preparation of membranes

Mouse brains were dissected to separate specific areas. Mouse cerebellum, hippocampus, cortex and striatum were homogenized

in a homogenization buffer containing 25 mM Tris–HCl pH=7.5, 25 mM NaCl, 5 mM EGTA, 2.5 mM EDTA, 2 mM NaPP, 4 mM PNFF, 1 mM Na_3VO_4, 1 mM PMSF, 20 μg/ml leupeptin, 50 μg/ml aprotinin, 0.1% SDS. The homogenate was centrifuged at 9000×g for 15 min at 4 °C and the low speed pellet was discarded. The microsomal membranes were obtained from the supernatant of the 9000×g spin by centrifugation at 100,000×g for 1 h at 4 °C. Microsomes were resuspended in homogenization buffer and stored at −80 °C. Protein concentration of the microsomal fraction was quantified using a protein assay kit (Bio Rad Laboratories, Milan, Italy). Skeletal muscle microsomes were prepared from the posterior leg of mice. Cardiac microsomes were prepared from the entire heart, cleaned from large vessels.

Western blot analysis

Membrane homogenates (100 μg) made from cerebellum, hippocampus, cortex and striatum regions of control and antisense-treated mice and membrane from skeletal and cardiac muscle were separated on 6% SDS-PAGE and transferred onto nitrocellulose membranes (180 min at 80 V) using standard procedures. Membrane were blocked in PBST (PBS containing 0.1% Tween) containing 5% nonfat dry milk for 90 min. Following washings, blots were incubated overnight at 4 °C with specific antibodies against RyR1, RyR2 or RyR3 (1:3000 dilution), a kind gift of Prof. Sorrentino from the Department of Neuroscience, University of Siena, Italy. After being washed with PBS containing 0.1% Tween, the nitrocellulose membrane was incubated with goat anti-rabbit horseradish peroxidase–conjugated secondary antisera (1:10,000) and left for 1 h at room temperature. Blots were then extensively washed according to the manufacturer's instruction and developed using enhanced chemiluminescence detection system (Pierce, Milan, Italy). Exposition and developing time used was standardized for all the blots. Densitometric analysis of scanned images was performed on a Macintosh iMac computer using the public domain NIH Image program. Measurements in control samples were assigned a relative value of 100%.

Immunoprecipitation of RyR1, RyR2, RyR3

Immunoprecipitation was carried out on 1 ml of microsomal fraction containing 200 μg proteins by incubation for 2 h at 4 °C with 10 μg of specific antibodies against RyR1, RyR2 or RyR3. All tubes then received 20 μl of 25% (v/v) Protein G-Agarose (Santa Cruz Biotechnology, CA, USA) and incubated for further 2 h at 4 °C. Pellets were collected by centrifugation at 1000×g for 5 min at 4 °C, washed three times with homogenization buffer. Pellets were finally resuspended in 40 μl electrophoresis sample buffer, boiled for 5 min, and samples were processed by SDS-PAGE.

Statistical analysis

All experimental results are given as the mean±S.E.M. An analysis of variance (ANOVA), followed by Fisher's protected least significant difference procedure for post hoc comparison, were used to verify significance between two means. Data were analyzed with the StatView software for the Macintosh. P values of less than 0.05 were considered significant.

RESULTS

Effect of aODNs on RyR1, RyR2 and RyR3 protein levels

Mice were treated with the aODNs on days 1, 2 and 3. On day 4, 18 h after the last i.c.v. injection, mice were killed and the cerebellum, cortex, striatum and hippocampus were dissected and examined for the protein levels of

RyR1, RyR2 and RyR3 in comparison with mice treated with dODN using specific antisera developed against each RyR isoform. Fig. 1A shows a representative immunoblot where three distinct prominent protein bands of approximately 500 kDa were observed which display a tissue specificity of expression. The anti-RyR1 antiserum selectivity was demonstrated by a specific interaction with RyR1 proteins present in the sarcoplasmic reticulum of skeletal muscle whereas the anti-RyR2 antiserum recognized the RyR2 proteins in cardiac muscle. RyR2 and RyR3 proteins were detected in all brain areas investigated. Conversely, RyR1 isoform was not detected in mouse cortex (Fig. 1A).

A statistically significant reduction of the expression of RyR1, RyR2 and RyR3 after aODN treatment in comparison with mice treated with the corresponding dODN was observed (Fig. 1A). Densitometric analysis of all samples revealed that every aODN treatment decreased expression of the corresponding receptors in all cerebral areas investigated (Fig. 1B).

The dODN did not significantly change the immunoreactivity when compared with that of naïve animals (data not shown). Immunoprecipitation experiments also produced similar results as illustrated in Fig. 2.

Immunoblots were re-probed for a protein considered to be not regulated as β-actin and no significant density difference was revealed for this protein between samples from the RyR1, RyR2 and RyR3 down-regulated brain region (data not shown).

Cross-reactivity of the primary antibodies used was excluded.

Role of RyR1, RyR2 and RyR3 on muscarinic antinociception in a thermal pain condition

Pretreatment with an aODN against RyR1 (0.5–5 nmol per mouse per i.c.v. injection) dose-dependently prevented the physostigmine-induced increase of pain threshold in comparison with dODN-treated mice ($F_{(4,75)}5.275$; $P<0.001$) (Fig. 3A). A shift to the right of the dose-response curve of physostigmine after anti-RyR1 pretreatment (5 nmol per mouse i.c.v.) was observed (Fig. 3B). By contrast, knockdown of RyR2 (0.5–5 nmol per mouse per i.c.v. injection) never modified the antinociceptive effect produced by physostigmine (0.15 mg kg^{-1} s.c.) administration (Fig. 4). Administration of an aODN against RyR3 (0.5–5 nmol per mouse per i.c.v. injection) prevented physostigmine antinociception ($F_{(4,70)}7.591$; $P<0.001$) (Fig. 5A). aODN pre-treatment shifted to the right the dose-response curve of physostigmine (Fig. 5B).

Pretreatment with an aODN against RyR1 (5 nmol per mouse per i.c.v. injection) and against RyR3 (5 nmol per mouse i.c.v.) did not alter the mouse pain threshold in comparison with dODN-treated animals (Figs. 3, 5).

dODN-pretreatment did not modify the mouse pain threshold in comparison with DOTAP- and saline-treated animals and with naïve animals (data not shown).

The antagonistic effect on physostigmine antinociception produced by the anti-RyR1 and anti-RyR3 treatments
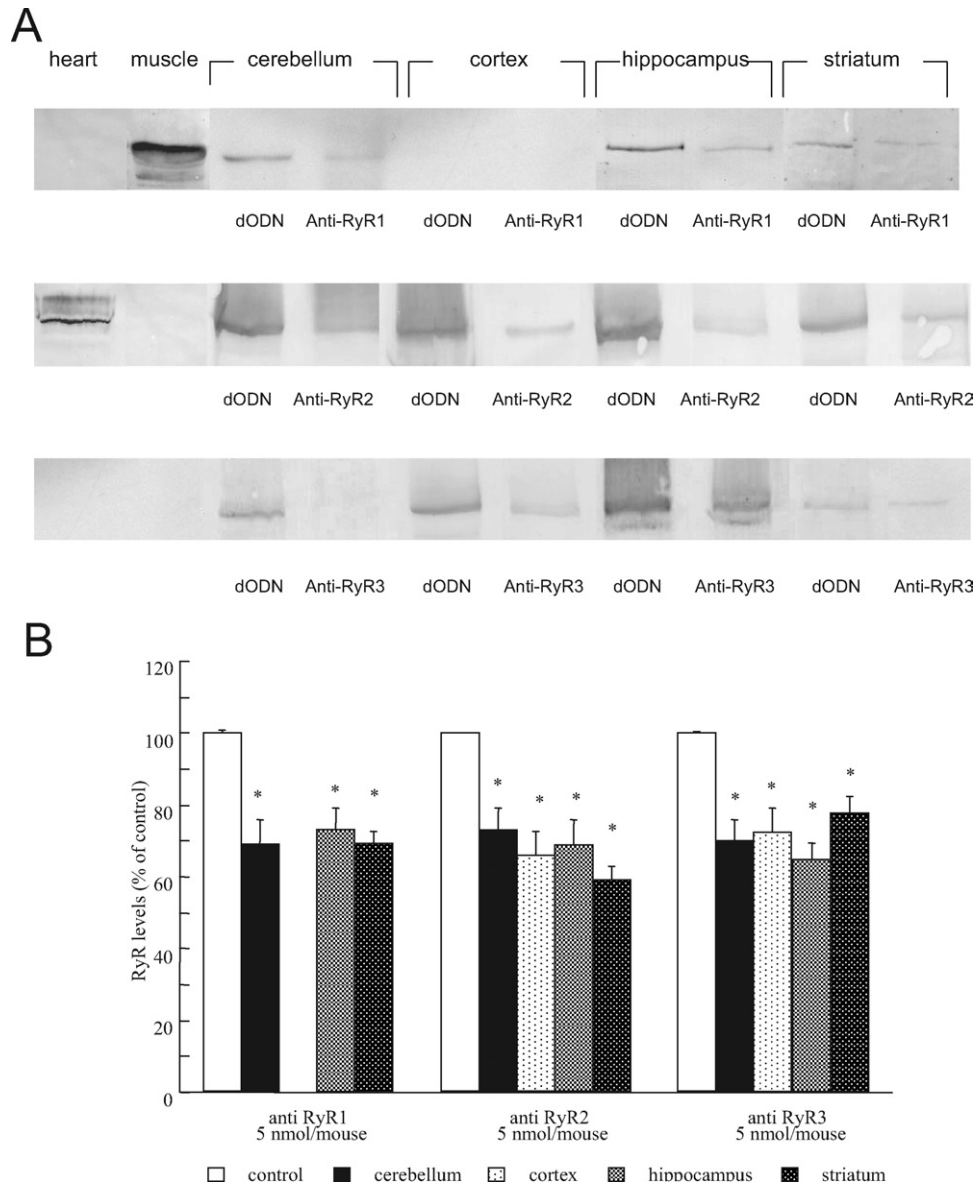
Fig. 1. Reduction of RyR1, RyR2 and RyR3 protein expression in mouse muscle, heart, cerebellum, cortex, hippocampus and striatum by aODN treatment in comparison with corresponding dODN-treated mice. (A) Samples (100 μg protein/lane) of microsomal fractions of mouse cerebellum, cortex, hippocampus and striatum, and samples of hearth (20 μg protein/lane) and muscle (50 μg protein/lane) were resolved on 6% SDS-PAGE, transferred to nitrocellulose and probed with RyR1, RyR2 or RyR3 antibody. (B) Densitometric quantitation of immunoreactive protein expressed relative to control. Data are expressed as mean±S.E.M. of band intensities from each of the four groups (n=7 per group). * P<0.05 versus control.

was reduced 48 h after the last i.c.v. injection and disappeared 7 days after the end of the treatment (Fig. 6).

Physostigmine antinociception was completely prevented by the nonselective RyR antagonist ryanodine (0.06 nmol per mouse i.c.v.) (F(9,63)5.107; P<0.001). By contrast, nonmuscarinic antinociception such as that induced by diphenhydramine (20 mg kg^{-1} s.c.), pyrilamine (20 mg kg^{-1} s.c.) and baclofen (4 mg kg^{-1} s.c.), was unmodified by ryanodine administration (Fig. 7).

dODN-pretreatment did not modify the mouse sensitivity to the physostigmine antinociceptive effect in comparison with naïve, DOTAP- and saline-treated mice (data not shown).

Role of RyR1, RyR2 and RyR3 on muscarinic antinociception in an inflammatory pain condition

Ryanodine (0.06 nmol per mouse i.c.v.) completely antagonized the antinociception induced by physostigmine (0.15 mg kg^{-1} s.c.) (F(5,54)6.235; P<0.001) in the mouse abdominal constriction test. Lower doses (0.01 and 0.03 nmol per mouse) were ineffective (Fig. 8A). Ryanodine 0.06 nmol per mouse, when administered alone, did not modify the mouse pain threshold in comparison with saline-treated mice (Fig. 8A).

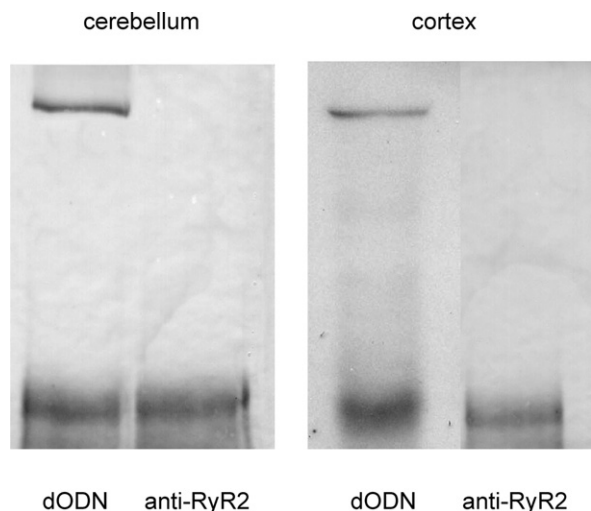Pretreatment with anti-RyR1 (5 nmol per mouse i.c.v.) or anti-RyR3 (5 nmol per mouse i.c.v.) significantly

cerebellum   cortex

dODN   anti-RyR2   dODN   anti-RyR2

Fig. 2. Reduction of RyR2 protein expression in mouse cortex and cerebellum by anti-RyR2 treatment in comparison with dODN-treated mice. Mouse cortex and cerebellum microsomal membranes were immunoprecipitated with the specific antibody against RyR2 and the immunoprecipitates, corresponding to 100 μg of original extract, were resolved on 6% SDS-PAGE, transferred to nitrocellulose and probed with RyR2 antibody.

prevented the physostigmine-induced antinociception ($F(4,45)11.5$; $P<0.001$). In RyR2 knockdown mice, the physostigmine antinociception remained unmodified in comparison with control group (Fig. 8B). Anti-RyR1, anti-RyR2 and anti-RyR3 treatments did not alter the mouse pain threshold in comparison with d-ODN group (data not shown).

Effect of treatments on mouse motor coordination and spontaneous mobility

The aODN, at the highest effective doses, were tested in order to assess their effect on mouse behavior. At the highest doses employed the aODNs did not induce any alteration of animals' gross behavior or side effects such as tremors and convulsions and/or death.

Mice pretreated with anti-RyR1 (5 nmol per mouse i.c.v.), anti-RyR2 (5 nmol per mouse i.c.v.), anti-RyR3 (5 nmol per mouse i.c.v.) and dODN (5 nmol per mouse i.c.v.) were evaluated for motor coordination by use of the rota-rod test, and for spontaneous mobility and inspection activity by use of the hole board test. The endurance time, evaluated before and 15, 30 and 45 min after the beginning of the rota-rod test, showed the lack of any impairment in the motor coordination of animals pretreated with aODNs in comparison with dODN group (Table 1). The spontaneous mobility (Fig. 9A) as well as the inspection activity (Fig. 9B) of mice, expressed as counts in 5 min, was unmodified by pretreatment with aODNs in comparison with dODN group. D-Amphetamine, used as positive control, significantly increased both parameters evaluated.

DISCUSSION

The involvement of RyR1, -2, and -3 in muscarinic analgesia was investigated in a condition of acute thermal and inflammatory nociception in mice. To investigate the role played by RyR isoforms an antisense strategy was used. The knockdown of RyR subtypes by administration of aODNs was necessary since mice missing the RyR1 and RyR2 gene products die early during embryonic development. Mice carrying a targeted disruption of the RyR1 gene show complete loss of the skeletal muscle excitation-contraction coupling and die perinatally due to respiratory failure (Takeshima et al., 1995). Similarly to that observed in RyR1 knockout mice, generation of mice carrying a targeted disruption of the RyR2 gene indicates a pivotal role of this isoform not only in cardiac excitation-contraction coupling, but also during myocardial development, as RyR2 knockout mice die at embryonic day 10 and show morphological abnormalities in the heart tube (Takeshima et al., 1998). These observations ruled out the possibility of
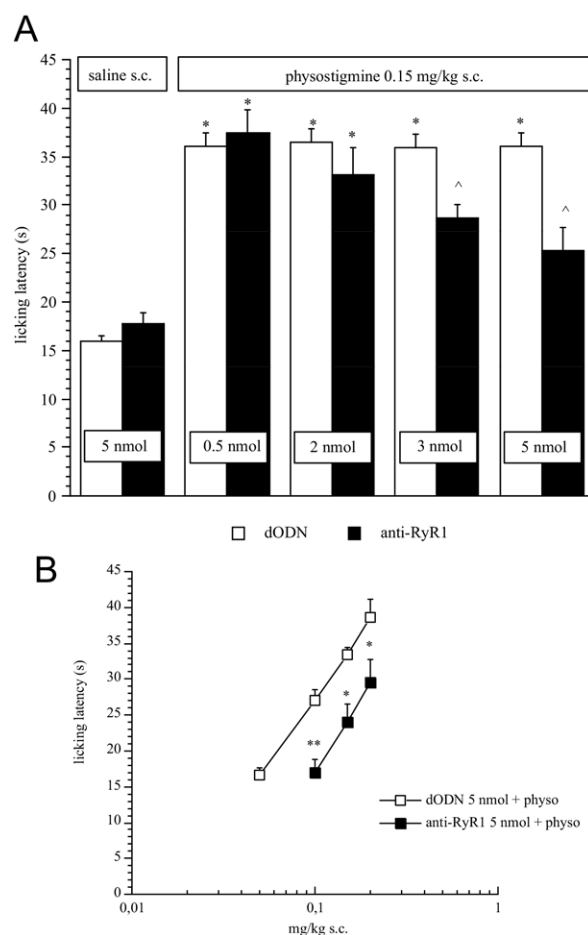

Fig. 3. (A) Prevention by an aODN against RyR1 subtype (anti-RyR1, 0.5–5 nmol per mouse i.c.v.) of physostigmine (0.15 mg kg^{-1} s.c.) -induced antinociception in the mouse hotplate test. The licking latency values were recorded 30 min after cholinomimetic administration. ODNs were administered once daily for 3 days. The test was performed 18 h after the last injection. Vertical lines represent S.E.M. * $P<0.001$ in comparison with control group (dODN+saline-treated mice); ^ $P<0.05$ in comparison with dODN+physostigmine-treated mice. (B) Shift to the right of the physostigmine dose-response curve by pretreatment with anti-RyR1 5 nmol per mouse ic.v. Vertical lines represent S.E.M. * $P<0.05$ in comparison with dODN+physostigmine-treated mice.
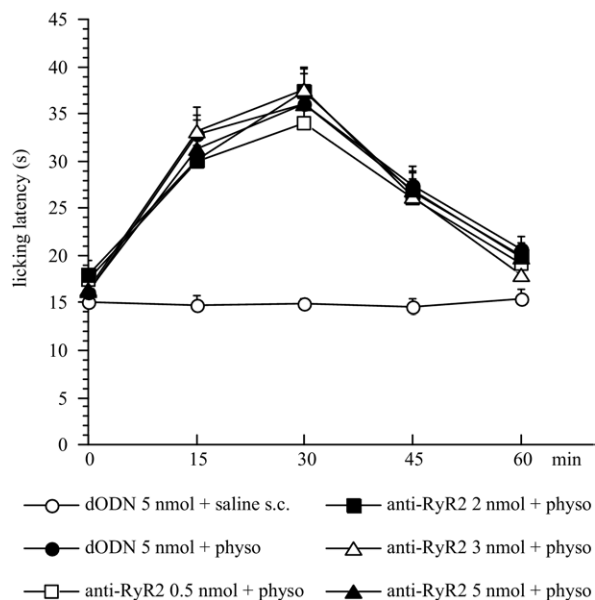
Fig. 4. Lack of effect of an aODN against RyR2 subtype (anti-RyR2, 0.5–5 nmol per mouse i.c.v.) on physostigmine (0.15 mg kg^{-1} s.c.) -induced antinociception in the mouse hotplate test. ODNs were administered once daily for 3 days. The test was performed 18 h after the last injection. Vertical lines represent S.E.M. * $P<0.05$ in comparison with control group (dODN+saline-treated mice).

using RyR1 and RyR2 knockout mice for behavioral studies.

The presence of the three RyR isoforms at supraspinal levels was demonstrated by Western blotting experiments. In particular RyR2 and RyR3 isoforms were present in microsomes from mouse cerebellum, hippocampus, cortex and striatum. RyR1 was present in cerebellum, hippocampus and striatum, but it was not detected in mouse cortex. These results are in agreement with previous studies in murine brain (Giannini et al., 1995).

The capability of the aODN treatment to knock down RyR protein levels was demonstrated by immunoblotting and immunoprecipitation experiments where the levels of RyR1, RyR2 and RyR3 from microsomes prepared from cerebellum, hippocampus, cortex and striatum of animals treated with the corresponding aODN in comparison with the dODN-treated mice were determined. A selective decrease of RyR1, RyR2 and RyR3 protein levels was evidenced in all cerebral areas investigated by using selective antibodies for each RyR isoform.

RyRs are distributed not only in the CNS but also at a peripheral level. RyRs are present in the skeletal and cardiac muscle where their activation represents a crucial step for muscle contraction (Rossi and Sorrentino, 2002). To avoid the possible appearance of peripheral effects that could lead to a misinterpretation of the results obtained, the aODNs used were administered directly into the cerebral ventricles.

The administration of an aODN against the type 1 RyR prevented the increase of the pain threshold induced by physostigmine and shifted to right the physostigmine dose-response curve demonstrating the importance of the re-

ceptor-mediated activation of RyR1 to obtain supraspinal muscarinic antinociception. The involvement of RyR1 in the modulation of the pain threshold at peripheral and spinal level has been hypothesized. The intraplantar and spinal administration of *Buthus martensi* Karsch, a scorpion active polypeptide endowed with RyR1 agonistic properties, produced antinociception in rats (Tan et al., 2001). Present results confirm literature data and give information about the involvement of RyR1 in the modulation of the pain threshold at the supraspinal level.

Similarly to RyR1, the selective knockdown of the type 3 RyR antagonized physostigmine-induced increase of the pain threshold and shifted to the right the dose-response curve of the cholinomimetic drug. These data indicate the importance of the muscarinic receptor-mediated RyR3 activation in the induction of cholinergic analgesia. RyR3 appears to have a prominent role in muscarinic analgesia, in
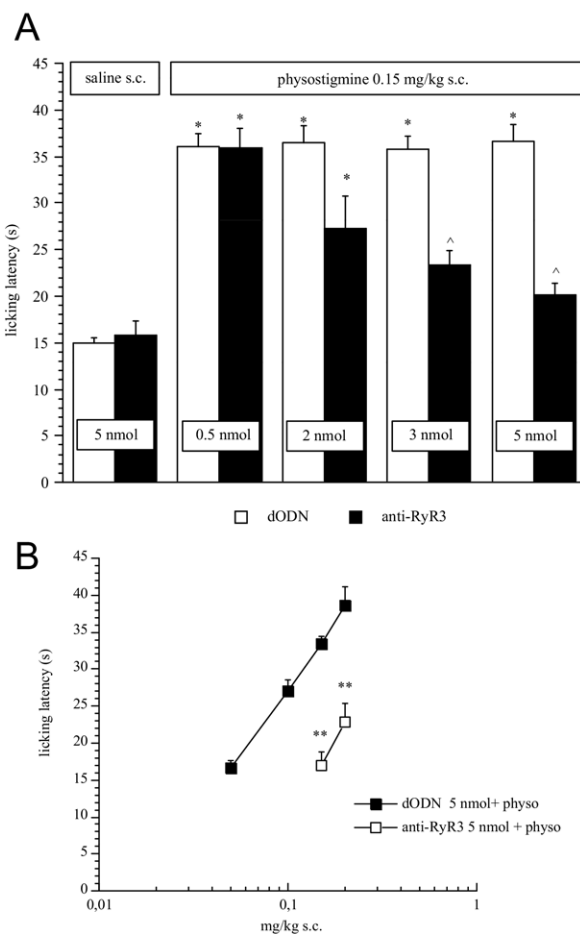

Fig. 5. (A) Prevention by an aODN against RyR3 subtype (anti-RyR3, 0.5–5 nmol per mouse i.c.v.) of physostigmine (0.15 mg kg^{-1} s.c.) -induced antinociception in the mouse hotplate test. The licking latency values were recorded 30 min after cholinomimetic administration. ODNs were administered once daily for 3 days. The test was performed 18 h after the last injection. Vertical lines represent S.E.M. * $P<0.001$ in comparison with control group (dODN+saline-treated mice); ^ $P<0.05$ in comparison with dODN+physostigmine-treated mice. (B) Shift to the right of the physostigmine dose-response curve by pretreatment with anti-RyR3 5 nmol per mouse ic.v. Vertical lines represent S.E.M. ** $P<0.01$ in comparison with dODN+physostigmine-treated mice.
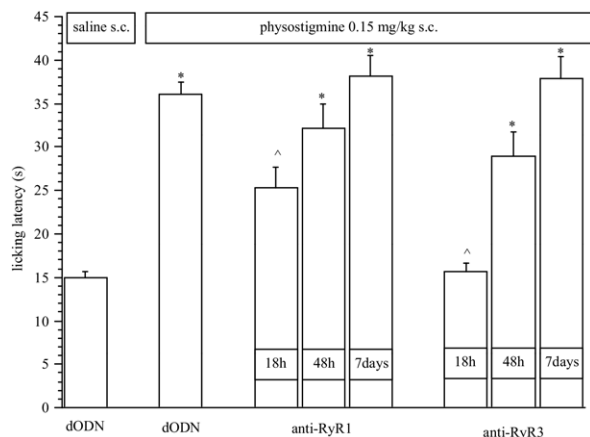
Fig. 6. Lack of effect of anti-RyR1 and anti-RyR3 treatment (5 nmol per mouse i.c.v.) on physostigmine (0.15 mg kg^{-1} s.c.) -induced antinociception in the mouse hotplate test 48 h and 7 days after the end of the ODN-treatment. The licking latency values were recorded 30 min after cholinomimetic administration. Vertical lines represent S.E.M. * $P < 0.001$ in comparison with control group (dODN+saline-treated mice). ^ $P < 0.05$ in comparison with dODN+physostigmine-treated mice.

comparison with RyR1, since an antagonistic effect of greater intensity was seen in RyR3 knockdown animals. This difference might be subsequent to a wider distribution of RyR3 in the brain. In particular, RyR3 are present in the cortex, a cerebral area involved in the modulation of pain threshold where M1 receptors are highly expressed whereas RyR1 are not detected. A role of RyR3 in the modulation of physiological processes in the CNS has been hypothesized. Mutant mice lacking RyR3 exhibit deficits of contextual fear conditioning, synaptic plasticity and spatial learning (Balschun et al., 1999; Kouzu et al., 2000). Present results extend the knowledge of the physiological role of RyR3 at the supraspinal level and represent the first
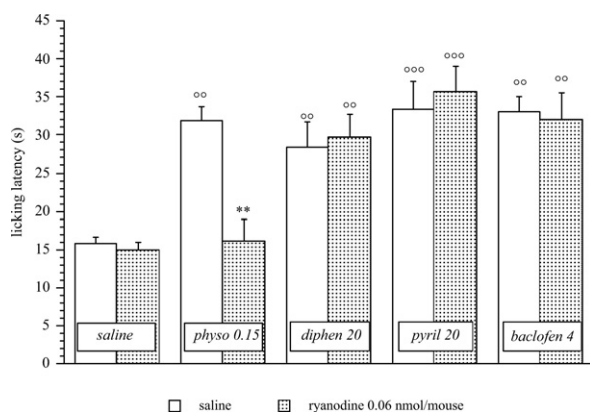

Fig. 7. Prevention by ryanodine (0.06 nmol per mouse i.c.v.) of physostigmine (0.15 mg kg^{-1} s.c.) -induced antinociception in the mouse hotplate test. Lack of effect on diphenhydramine (20 mg kg^{-1} s.c.), pyrilamine (20 mg kg^{-1} s.c.) and baclofen (4 mg kg^{-1} s.c.) -induced antinociception. The licking latency values were recorded 30 min after physostigmine, 15 min after diphenhydramine and pyrilamine, and 45 min after baclofen administration. Vertical lines represent S.E.M. °° $P < 0.01$, °°° $P < 0.001$ in comparison with saline, ** $P < 0.01$ in comparison with physostigmine-treated mice.
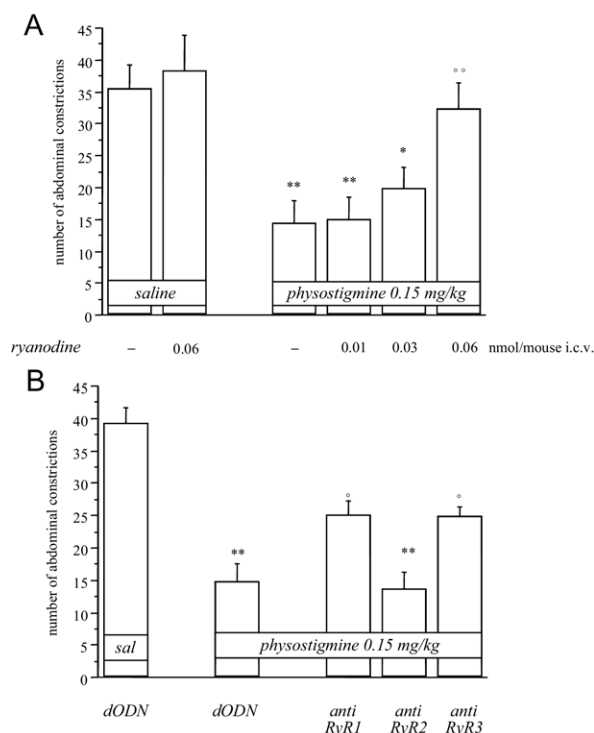

Fig. 8. (A) Prevention by ryanodine (0.06 nmol per mouse i.c.v.) of physostigmine (0.15 mg kg^{-1} s.c.) -induced antinociception in the mouse abdominal constriction test. Vertical lines represent S.E.M. °° $P < 0.01$ in comparison with physostigmine-treated mice, ** $P < 0.01$, * $P < 0.05$ in comparison with saline-treated mice. (B) Prevention by an aODN against RyR1 subtype (anti-RyR1, 5 nmol per mouse i.c.v.) and an aODN against RyR3 subtype (anti-RyR3, 5 nmol per mouse i.c.v.) of physostigmine (0.15 mg kg^{-1} s.c.) -induced antinociception in the mouse abdominal constriction test. ODNs were administered once daily for 3 days. The test was performed 18 h after the last injection. Vertical lines represent S.E.M. ** $P < 0.01$ in comparison with control group (dODN+saline-treated mice); ° $P < 0.05$ in comparison with dODN+physostigmine-treated mice.

indication of involvement of RyR3 in the modulation of pain perception.

In RyR1 and RyR3 knockdown animals, a partial block of physostigmine antinociception was observed indicating that both receptor subtypes are involved in muscarinic increase of pain threshold. As further confirmation, the administration of the non-selective RyR antagonist ryanodine produced a complete antagonism of physostigmine antinociception. A lack of effect of ryanodine on non-mus-

Table 1. Lack of effect by anti-RyR1, anti-RyR2 and anti-RyR3 on the motor coordination evaluated in the mouse rota rod test

Treatment	Number of falls in 30 s			
	Pretest	15 min	30 min	45 min
dODN 5 nmol	3.7±0.7	1.8±0.3	1.1±0.3	0.9±0.2
Anti-RyR1 5 nmol	4.0±1.1	1.9±0.6	1.0±0.4	0.8±0.3
Anti-RyR2 5 nmol	4.0±0.9	2.0±0.5	0.9±0.3	0.6±0.2
Anti-RyR3 5 nmol	3.6±0.7	1.5±0.3	1.0±0.2	0.9±0.3

ODNs were administered once daily for 3 days. The rota rod test was performed 18 h after the last injection.
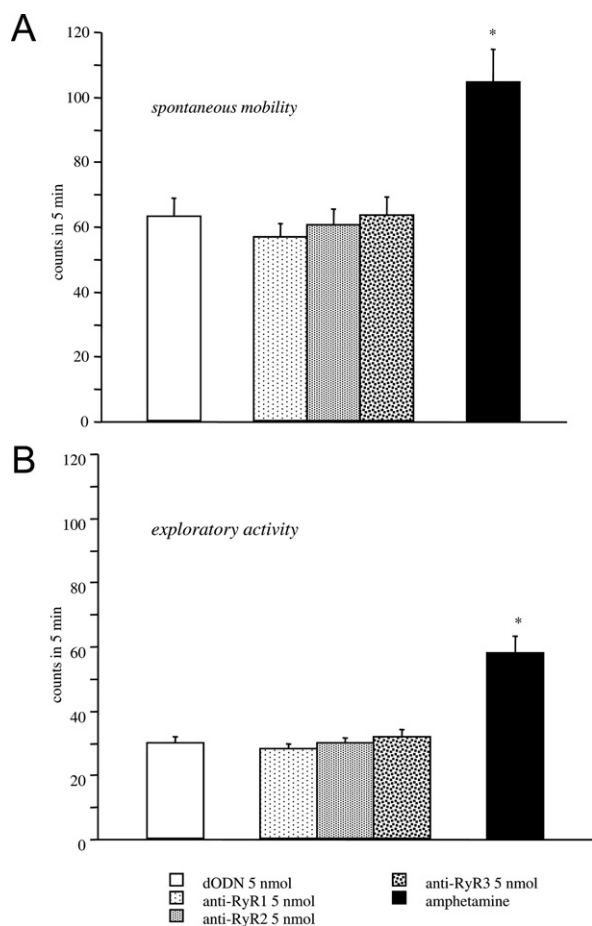
Fig. 9. Lack of effect of anti-RyR1, anti-RyR2 and anti-RyR3 treatment (5 nmol per mouse i.c.v.) on mouse spontaneous mobility (A) and exploratory activity (B) evaluated in the mouse hole board test. ODNs were administered once daily for 3 days. The test was performed 18 h after the last injection. Amphetamine was administered at the dose of 2 mg kg^{-1} s.c.; vertical lines represent S.E.M.

carinic antinociception, such as that induced by diphenhydramine, pyrilamine and baclofen, ruled out the possibility of a unspecific effect produced by the RyR antagonist on physostigmine antinociception.

The inhibition of physostigmine analgesia by anti-RyR1 and anti-RyR3 decreased 48 h after the end of the antisense treatment and disappeared 7 days after the last i.c.v. injection. These data indicate a lack of irreversible damage or toxicity on cerebral structures caused by the aODNs. Pretreatment with anti-RyR1 and anti-RyR3, in the absence of co-administration of cholinomimetic drugs, did not modify the mouse pain threshold, showing the lack of a hyperalgesic effect induced by the aODN treatment. The lack of any alteration of the pain threshold in the mouse hotplate test was also observed in mutant mice lacking RyR3 (Kouzu et al., 2000). Therefore, the prevention of physostigmine antinociception cannot be attributable to a direct effect on the pain threshold induced by the aODNs. Furthermore, the dODN, used as control ODN treatment, did not modify cholinergic antinociception in comparison with naive or saline- and vector-i.c.v.-injected mice. This

observation ruled out the possibility that the antagonism exerted by the aODNs may have resulted from a sequence-independent action on cerebral structures.

Present results indicate that the release of Ca^{2+} from intracellular stores produced by RyR1 and RyR3 activation is necessary to induce muscarinic antinociception. It should also be noted that an increased intracellular calcium mobilization through RyR activation has been demonstrated after stimulation of muscarinic receptors. Carbachol-evoked increase of intracellular calcium concentration in cerebellar granule cells was inhibited by ryanodine (Simpson et al., 1996). Activation of muscarinic acetylcholine receptors in NG108-15 neuroblastoma×glioma cells produces cyclic ADP-ribose, a known endogenous modulator of RyR, which may upregulate the release of calcium from the RyRs (Higashida et al., 1997, 2001). Carbachol-induced contractions of rat stomach fundus strips were reduced by ryanodine and thapsigargin (Smaili et al., 2001). Similarly, ryanodine blocked the early contraction component increased by carbachol in guinea-pig ventricular myocytes (Protas et al., 1998).

Pretreatment with an aODN against the type 2 RyR did not antagonize the antinociception induced by physostigmine either in the hotplate or in the abdominal constriction test, suggesting the lack of involvement of this receptor subtype in muscarinic analgesia. RyR2 represents the predominant isoform in the CNS found in widespread brain regions (McPherson and Campbell, 1993; Giannini et al., 1995). Some studies indicate that RyR2, similarly to RyR3, is involved in the modulation of memory processes. An increase of the RyR2 mRNA and protein was found in rats trained in an intensive water maze task (Zhao et al., 2000). In spite of this evidence, the absence of a major role of RyR2 in cholinergic analgesia was evidenced in our experimental conditions. It should be noted that RyR2 mRNA is poorly expressed in the thalamus (Zhao et al., 2000), a brain area highly involved in pain perception. This observation led to the plausible supposition that the RyR2 isoform is not endowed with a prominent role in the modulation of the pain threshold. The lack of antagonism by the anti-RyR2 aODN on cholinergic antinociception as a consequence to inefficient knockdown of the RyR2 protein by the antisense treatment can be ruled out. Western blotting and immunoprecipitation experiments performed on RyR2 knockdown mice revealed a reduction of the RyR2 protein levels in all cerebral areas investigated.

The receptor-mediated activation of the muscarinic system can induce several side effects. It is widely known that cholinomimetic drugs can produce the typical cholinergic symptomatology (tremors, sialorrhea, diarrhea, lacrimation, etc.). Cytosolic Ca^{2+} regulates numerous neuronal functions (Berridge, 1998) and, therefore, a variation of intracellular Ca^{2+} contents can induce behavioral side effects. aODNs were tested before the hotplate and abdominal constriction tests were performed, to make sure that treatments did not alter the normal locomotor behavior of the mice. An influence of the substances used on spontaneous mobility has been excluded by using the hole board test. Not only a modified spontaneous mobility but also

altered motor coordination could lead to a misinterpretation of the results obtained. A rota rod test was, therefore, performed and any alteration of the motor activity induced by aODN administration at the highest doses used was excluded. The results of the rota rod and hole board tests were of particular relevance since it has been observed that deletion of RyR3 induces an increased speed of loco-motion and a mild tendency to circular running (Balschun et al., 1999).

Seen as a whole, our data evidence that the selective activation of RyR1 and -3 is required in the induction of cholinergic supraspinal analgesia in mice in a condition of acute thermal and inflammatory nociception. Furthermore, the lack of a prominent role of the RyR2 in the increase of pain threshold induced by physostigmine has also been observed.

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