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A gene-specific cerebral types 1, 2, and 3 RyR protein knockdown induces an antidepressant-like effect in mice

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Abstract

Elevation of baseline intracellular calcium levels was observed in platelets or lymphoblasts of patients with bipolar affective disorders suggesting an altered intracellular Ca^{2+} homeostasis in the pathophysiology of mood disorders. The role of supraspinal endoplasmic ryanodine receptors (RyRs), which allow mobilization of intracellular Ca^{2+} stores, in the modulation of depressive states was, then, investigated. Ryanodine and FK506 reduced the immobility time in the mouse forced swimming test showing an antidepressant-like profile comparable with that produced by amitriptyline and clomipramine. We generated types 1, 2, and 3 RyR knockdown mice by using selective antisense oligonucleotides (aODN) to investigate the role of each RyR isoform. A gene-specific cerebral RyR protein level reduction in knockdown animals was demonstrated by immunoblotting, immunoprecipitation, and immunohistochemical experiments. Repeated intracerebroventricular administration of aODNs complementary to the sequence of the types 1, 2, or 3 RyR produced an antidepressant-like response in the forced swimming test. The aODN-induced reduction of immobility time was temporary and reversible and did not impair motor coordination, spontaneous mobility, and exploratory activity. These findings identify cerebral RyRs as critical targets underlying depressive states and should facilitate the comprehension of the pathophysiology of mood disorders and help developing of new therapeutical strategies.

Keywords: antidepressant, antisense oligonucleotide, depression, forced swimming test, intracellular Ca^{2+}, ryanodine receptor.

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Calcium ions are recognized to play an important role in the regulation of neuronal functions. An altered Ca^{2+} homeostasis might also be involved in the pathophysiology of neuronal disorders, such as mood disorders. Some investigations reported that baseline intracellular calcium levels are elevated in platelets or lymphoblasts of patients with bipolar affective disorders (Dubovsky *et al.* 1994; Emamghoreishi *et al.* 1997).

The regulation of free intracellular Ca^{2+} is a complex, multifaceted process 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 (IP_3R), and the ryanodine receptor (RyR) (Foskett *et al.* 2007; Zalk *et al.* 2007).

Several studies have shown an overstimulated phosphoinositide signaling system in affective disorders. Results from depressed patients evidenced the existence of a greater sensitivity of inositol-phospholipid second messenger system (Bohus *et al.* 1996) and an altered phosphatidyl inositol 4,5-bisphosphate $(PIP)_2$ hydrolysis (Mikuni *et al.* 1991; Karege *et al.* 1996; Pacheco *et al.* 1996; Shimon *et al.* 1997; Pandey *et al.* 2001; Soares *et al.* 2001) in blood cells and postmortem brains. Furthermore, elevated IP_3 binding sites and expressed IP_3 receptor protein levels have been noted in platelets from depressed patients (Dwivedi *et al.* 1998; Rosel *et al.* 2000). More recently, the complete disappearance of depressive symptoms after addition of subcutaneous calcium heparin, an IP_3 receptor antagonist, to fluoxetine was observed in a patient suffering from a recurrent depression resistant to conventional antidepressant therapy (Maluquer *et al.* 2002).

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Abbreviations used: aODN, antisense oligonucleotide; dODN, degenerate oligonucleotide; FKBP12, FK506-binding protein 12; i.c.v, intracerebroventricular; IP_3R, inositol 1,4,5-trisphosphate receptor; PBS, phosphate-buffered saline; RT, room temperature; RyR, ryanodine receptor; SDS, sodium dodecyl sulfate.

Ryanodine receptors, similarly to IP_3R, modulates Ca^{2+} efflux from intracellular stores. In particular, while IP_3R is a key molecule for IP_3-induced Ca^{2+} release, RyR is important for Ca^{2+}-induced Ca^{2+} release (Foskett *et al.* 2007; Zalk *et al.* 2007). They also share a similar cerebral distribution (Giannini *et al.* 1995; Berridge 1998; Sorrentino and Rizzuto 2001) and appear to have a common origin (Sorrentino *et al.* 2000). The structure similarity of RyR and IP_3R proteins and the high level of sequence homology support this theory (Wagenknecht and Radermacher 1997; Galvan *et al.* 1999). 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 (Zalk *et al.* 2007). 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 are also detected in the CNS. Type 2 RyR represents the predominant cerebral isoform found in widespread brain regions; RyR3, the so-called brain isoform, is highly expressed in the CNS as well whereas, type 1 RyR has a limited central presence (McPherson and Campbell 1991; Giannini *et al.* 1995). The role of RyR channels in cell signaling pathways in the CNS is poorly understood and, in particular, little is known on their involvement in mood disorders. The aim of this study was to investigate the role of supraspinal endoplasmic types 1, 2, and 3 RyR in the modulation of depressive conditions by using the mouse forced swimming test, an animal model widely used to test the activity of antidepressant drugs. Mice missing the RyR1 and RyR2 gene products die early during embryonic development (Takeshima *et al.* 1995, 1998). Mice lacking both RyR1 and RyR3 were also generated. These double-mutant mice did not actively move after birth and died, most probably from respiratory failure, as was the case for RyR1-deficient mice (Ikemoto *et al.* 1997). These observations ruled out the possibility of using knockout mice for behavioral studies. To this purpose we generated knockdown animals through a selective inhibition of the expression of each RyR subtype in the CNS by using antisense oligonucleotides (aODN).

Materials and methods

Animals

Male Swiss albino mice (20–22 g) from the Morini (San Polo d'Enza, Italy) breeding farm were used. Fifteen mice were housed per cage (26 × 41 cm). 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 on at 7 AM. All experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health.

Intracerebroventricular injection technique

Intracerebroventricular (i.c.v.) administration was performed under ether anesthesia, as previously described (Galeotti *et al.* 2003). Briefly, during anesthesia, mice were grasped firmly by the loose skin behind the head. A 0.4 mm external diameter, hypodermic needle 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 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 into the right or left ventricle randomly. To ascertain that the drugs were administered exactly into the cerebral ventricle, some mice (20%) were injected with 5 μL of diluted 1 : 10 India ink and their brains examined macroscopically after sectioning. The accuracy of the injection technique was evaluated and the percentage of correct injections was determined to be 95%. Drug concentrations were prepared so that the necessary dose could be administered in a volume of 5 μL per mouse.

Forced swimming test

The forced swimming test used was the same as described by Porsolt *et al.* (1977). Briefly, mice were placed individually into glass cylinders (height 25 cm and diameter 10 cm) containing 6 cm of water maintained at 22–23°C and left there for 6 min. A mouse was judged to be immobile when it floated in the water, in an upright position, and made only small movements to keep its head above water. The duration of immobility was recorded during the last 4 min of the 6-min test. A decrease in the duration of immobility is indicative of an antidepressant-like effect; 12–15 mice per group were tested.

Hole-board test

The hole-board test consisted of a 40 cm square plane with 16 flush mounted cylindrical holes (3 cm diameter) distributed 4 by 4 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 photobeams, 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 (locomotor activity). Miniature photoelectric cells, in each of the 16 holes, recorded (counts in 5 min) the exploration of the holes (exploratory activity) by the mice; 12–15 mice per group were tested.

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 rpm. 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 3 and more than 6 falls in the pre-test were rejected (20%). The performance time was measured before (pre-test) and 15, 30, and 45 min after the beginning of the test; 12–15 mice per group were tested.

Preparation of membranes

Mouse brains were dissected to separate specific areas. Mouse cerebellum, hippocampus, and frontal cortex were homogenized in an 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 p-nitrophenyl phosphate, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 20 μg/mL leupeptin, 50 μg/ mL aprotinin, and 0.1% sodium dodecyl sulfate (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, and frontal cortex regions of control and antisense-treated mice and membrane homogenates from skeletal and cardiac muscle were separated on 6% SDS–polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (180 min at 80 V) using standard procedures. Membrane were blocked in phosphate-buffered saline (PBS) containing 0.1% Tween containing 5% non-fat 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 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 25°C. 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 (Apple, Florence, Italy) computer using the public domain NIH Image program (Bethesda, MD, USA). Measurements in control samples were assigned a relative value of 100%.

Immunoprecipitation of RyR1, RyR2, and 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, Santa Cruz, 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–polyacrylamide gel electrophoresis.

Tissue processing and immunocytochemistry

Mice were anesthetized with chloral hydrate 12% and perfused with *p*-formaldehyde 4% in 0.1 M phosphate buffer. The brains were removed and post-fixed in the same fixative for 4 h at 4°C. After post-fixation tissues were transferred to 0.1 M phosphate buffer containing sucrose 18% and then frozen in isopentane at −5°C. Floating sections (40 μm thick) were cut with cryostat CM1800 (Leica Mycrosystem, Milan, Italy). For antigen retrieval, the slides were incubated in 10 mM sodium citrate, pH 6, at 80°C for 30 min. Sections were rinsed with 0.1% Triton in PBS and incubated in blocking solution for 30 min at 25°C. Blocking solution contained 2% non-fat dry milk and 0.1% Triton in PBS. Sections were then incubated 48 h at 4°C with a primary polyclonal anti-RyR1, anti-RyR2, or anti-RyR3 (1 : 500). After rinsing in 0.1% Triton PBS, sections were incubated for 1 h at 25°C with diluted biotinylated secondary antibody solution (1 : 200) (Vector laboratories Inc., Burlingame, CA, USA). The sections were stained using the avidin–biotin peroxidase complex method with a Vectastain Kit (Vector laboratories Inc.) and then washed in PBS before development with 3-3′ diaminobenzidine (DAB) staining kit (Vector laboratories Inc.). Sections were rinsed with PBS and mounted on gelatinized glass slides. After dehydration in graded alcohol solutions, the sections were coverslipped with Pertex (Histo-line laboratories, Milano, Italy).

Antisense oligonucleotides

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*CCCCTGACATGTCC*C*C-3′; anti-RyR2: 5′-T*T*CGCCCGCATCAGCC*A*T-3′; anti-RyR3: 5′-A*G*ATGCTAATTGCATC*T*C-3′ (*indicates the phosphorothioate residues). A 18-mer fully degenerated ODN (dODN), 5′-N*N*NNNNNNNNNNNNN*N*N-3′ (where N is G, C, A, or T), was used as a control ODN ODNs were vehiculated intracellularly by an artificial cationic lipid (DOTAP; Sigma, Milan, Italy) to enhance both uptake and stability. Antisense ODNs (aODNs) or dODNs were pre-incubated 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.

Drug and oligo administration

The following drugs were used: ryanodine (Calbiochem, Milan Italy); D-amphetamine hydrochloride (De Angeli, Rome, Italy); FK506, amitriptyline, and clomipramine hydrochloride (Sigma). All drugs were dissolved in isotonic (NaCl 0.9%) saline solution. Doses and administration schedule for each compound were chosen on the basis of time-course and dose–response curves performed in our laboratory.

The forced swimming test was performed 15 min after ryanodine (0.001–0.1 nmol per mouse i.c.v.) and FK506 (0.12–37.3 nmol per mouse i.c.v.) administration, 30 min after clomipramine (25 μg per mouse i.c.v.) and amitriptyline (15 μg per mouse i.c.v.) injection. D-amphetamine (2 μg per mouse i.c.v.) was administered 15 min before the hole-board test.

Oligonucleotides were i.c.v. injected once daily on days 1, 2, and 3. Behavioral tests were performed on day 4, 18 h after the last i.c.v. injection of ODNs. To perform ODN time-course experiments, the

immobility time values were recorded on days 4, 5, and 10 corresponding to 24 h, 48 h, and 7 days after the end of the ODN treatment (5 nmol per mouse), respectively. In the hole board and rota-rod tests, ODNs were injected at the dose of 5 nmol per mouse i.c.v.

Brain areas to conduct western blotting and immunocytochemistry experiments were collected on day 4, 18 h after the last i.c.v injection of ODN (5 nmol per mouse i.c.v).

Statistical analysis

All experimental results are given as the mean ± SEM. Student's *t*-test or ANOVA followed by Bonferroni/Dunn *post hoc* test was used for statistical analysis.

Results

Effect of blockade of RyR-mediated pathway in the mouse forced swimming test

The i.c.v. administration of ryanodine, a selective RyR antagonist, decreased the immobility time values in the mouse forced swimming test. Ryanodine, at 0.001 nmol per mouse i.c.v., was devoid of any effect, whereas the doses of ryanodine of 0.01 and 0.03 nmol per mouse decreased the immobility time without reaching the statistical significance. The ANOVA on the retention session latencies revealed a significant group effect [$F(5,82)$ 5.784; $p < 0.0001$]. Bonferroni/Dunn multiple comparison analysis showed that mice receiving either 0.06 ($p < 0.05$) or 0.1 nmol ($p < 0.01$) per mouse i.c.v. induced a statistically significant antidepressant-like effect (Fig. 1a). Similarly, the i.c.v. administration of FK506 produced an antidepressant-like effect [$F(3,62)$ 3.978; $p < 0.001$]. The dose of 0.12 nmol per mouse i.c.v was ineffective whereas the statistical significance (Bonferroni/Dunn multiple comparison analysis) was reached at 12.4 nmol ($p < 0.05$) and 37.3 nmol ($p < 0.01$) per mouse i.c.v. (Fig. 1b). The intensity of the ryanodine and FK506 antidepressant-like effect was comparable with that produced by amitriptyline and clomipramine, used as reference drugs.

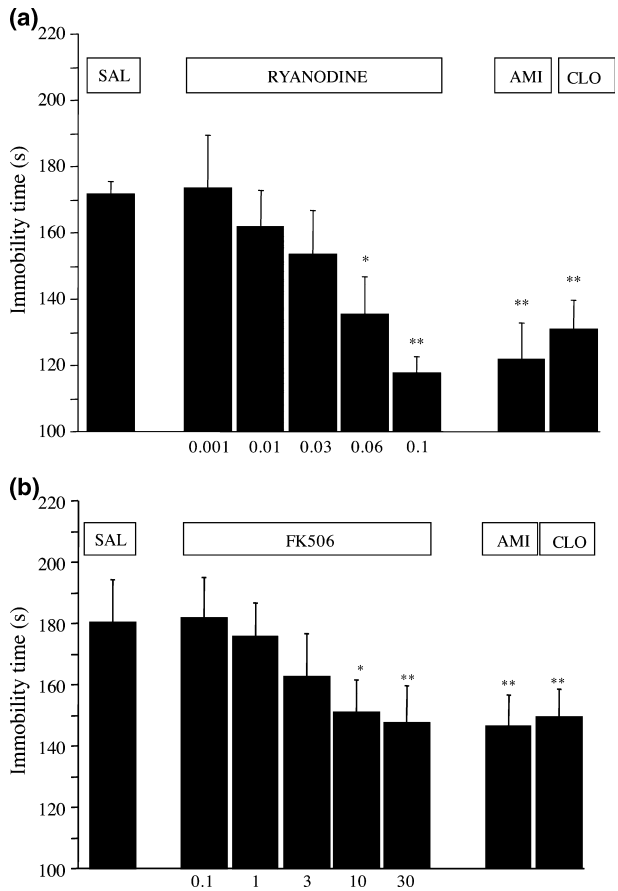

Fig. 1 Antidepressant-like effect produced by pharmacological modulation of RyR activity. Decrease by ryanodine (a) and FK506 (b) of the immobility time in the mouse forced swimming test. Doses of ryanodine and FK506 are expressed as nmol per mouse i.c.v. Vertical lines represent SEM. *$p < 0.05$ and **$p < 0.01$ in comparison with saline-treated mice. AMI, amitriptyline; CLO, clomipramine.

Reduction by aODN of cerebral RyR1, RyR2, and RyR3 protein levels

Cerebellum, frontal cortex, and hippocampus of RyR knockdown mice were dissected and examined for the protein levels of RyR1, RyR2, and RyR3 in comparison with mice treated with dODN (reference ODN) using specific antisera developed against each RyR isoform. 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. All three RyR isoforms were expressed in the cerebellum (data not shown) in agreement with previous studies in murine brain (Giannini *et al.* 1995). Figure 2(a and c) shows a representative immunoblot where three distinct prominent protein bands of approximately 500 kDa were observed which display a tissue specificity of expression. RyR2 and RyR3 proteins were detected in both cerebral cortex (Fig. 2a) and hippocampus (Fig. 2c). Conversely, RyR1 isoform was not detected in mouse cortex (Fig. 2a). A statistically significant reduction of RyR1, RyR2, and RyR3 protein levels in knockdown mice was observed. Densitometric analysis of all samples revealed that every aODN treatment decreased levels of the corresponding receptors in both cerebral cortex (Fig. 2a) and hippocampus (Fig. 2c). Immunoprecipitation experiments also produced similar results. A representative immunoblot showing the reduction of protein expression induced by anti-RyR2, taken as an example, in comparison with dODN-treated mice, in mouse cerebral cortex and hippocampus was illustrated, respectively, in Fig. 2b and d.

Immunoblots were re-probed for a protein considered to be not regulated as β-actin and no significant density difference

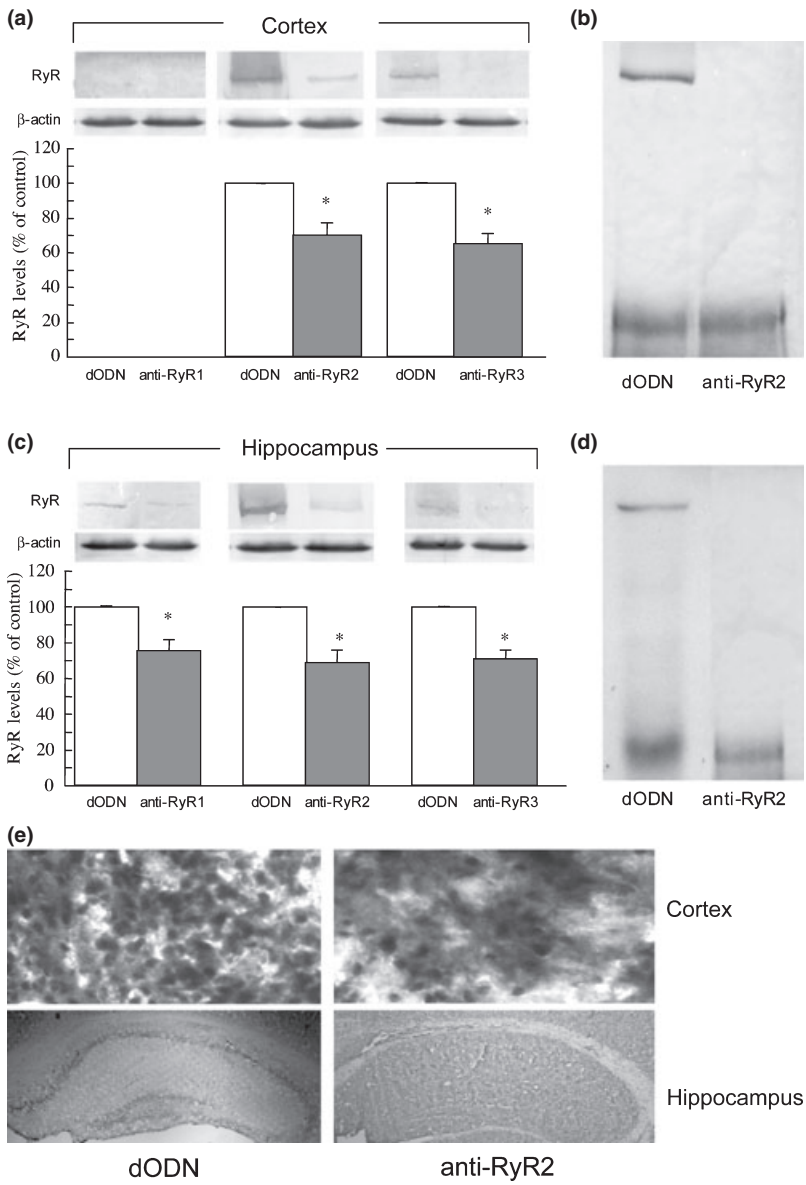

Fig. 2 Reduction of RyR1, RyR2, and RyR3 protein expression after aODN administration in RyR knockdown mouse brain. Protein level reduction was detected in mouse cortex (a) and hippocampus (c) in comparison with corresponding dODN-treated mice by using immunoblotting technique. The columns represent the densitometric quantitation of immunoreactive protein expressed relative to control. Data are expressed as mean ± SEM of band intensities ($n = 7$ per group); *$p < 0.05$ versus control (Student's t test). Representative immunoblots showing the reduction of RyR2 protein expression in mouse cortex (b) and hippocampus (d) by anti-RyR2 treatment in comparison with dODN-treated mice obtained in immunoprecipitation experiments. (e) Reduction of RyR2 protein expression in the mouse cortex and hippocampus of RyR2 knockdown mice. Representative photomicrographs for RyR2 immunoreactivity in cortical and hippocampal slices of control (dODN) and knockdown animals (anti-RyR2), obtained by immunocytochemistry experiments, were illustrated.

was revealed for this protein between samples from the RyR1, RyR2, and RyR3 down-regulated brain region (Fig. 2a and c).

Primary antibodies were developed against fusion proteins and extensively characterized (Giannini *et al.* 1995). Cross-reactivity of the primary antibodies used was further excluded by immunoprecipitation experiments.

Brain slices of treated and control mice were immunostained to show the localization and level of expression of RyRs. A lower immunostaining was observed in cerebellum, hippocampus, and frontal cortex of RyR knockdown animals in comparison with dODN-treated mice. Results obtained in anti-RyR2 knockdown animals, taken as example, were reported in Fig. 2e. This protein was detected in the mouse cortex and hippocampus of control animals (Fig. 2e). A reduction of protein expression was observed in RyR2 knockdown animals (Fig. 2e).

Effect of anti-RyR1, anti-RyR2, and anti-RyR3 in the mouse forced swimming test

Pre-treatment with an anti-RyR1 [$F(6,98)$ 5.712], anti-RyR2 [$F(6,101)$ 6.035], or anti-RyR3 [$F(8,123)$ 5.123] induced an antidepressant-like effect. The doses of 0.2 and 0.5 nmol per mouse i.c.v. were devoid of any effect. At 2 nmol per mouse i.c.v. anti-RyR2 (Fig. 3b) and anti-RyR3 (Fig. 3c) induced a statistically significant decrease in the immobility time of animals ($p < 0.05$, Bonferroni/Dunn multiple comparison analysis). The maximum antidepressant-like effect was obtained at 5 nmol per mouse i.c.v. for all three isoforms ($p < 0.01$, Bonferroni/Dunn multiple comparison analysis)

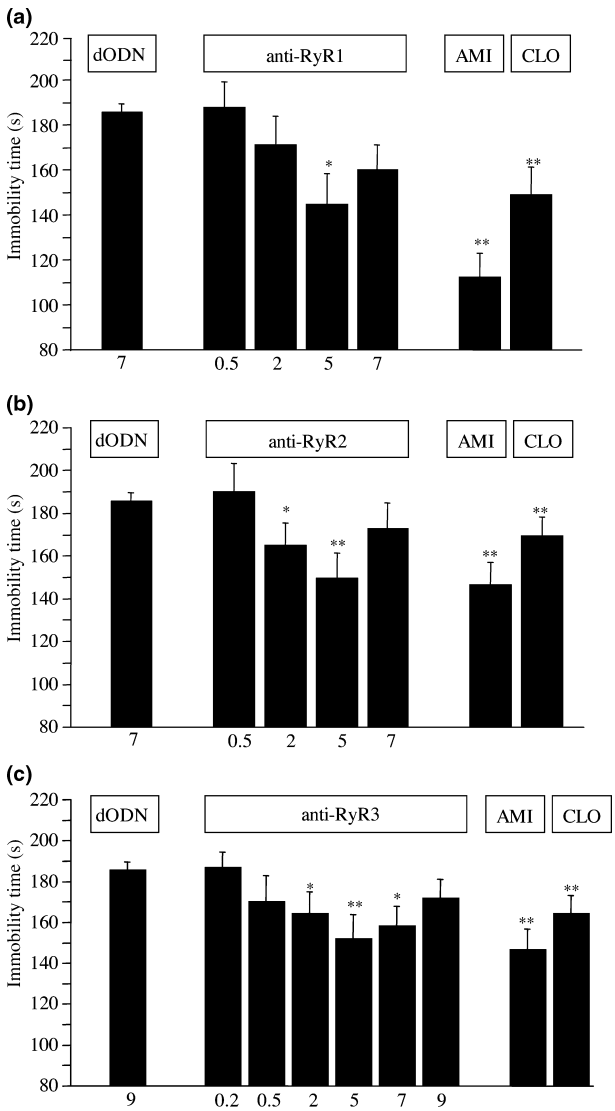

Fig. 3 Antidepressant-like effect produced by knockdown of RyR isoform proteins. Dose-dependent reduction by anti-RyR1 (a), anti-RyR2 (b), and anti-RyR3 (c) of the immobility time was detected in the mouse forced swimming test in comparison with amitriptyline (AMI) and clomipramine (CLO), used as reference drugs. aODN doses administered (nmol) were reported in each column. Vertical lines represent SEM; $*p < 0.05$ and $**p < 0.01$ in comparison with control mice.

(Fig. 3a, b, and c). At higher doses (7 pmol per mouse for anti-RyR1 and anti-RyR2; 9 pmol per mouse for anti-RyR3) the immobility time values returned to values comparable with that of control animals. The decrease in the immobility time induced by anti-RyRs was comparable with that induced by the antidepressant drugs amitriptyline and clomipramine, used as reference compounds, as shown in Fig. 3.

The antidepressant-like effect produced by anti-RyR1, anti-RyR2, and anti-RyR3 (5 nmol per mouse i.c.v.) was time dependent [$F(9,137)$ 5.131]. It peaked 18 h after the end of

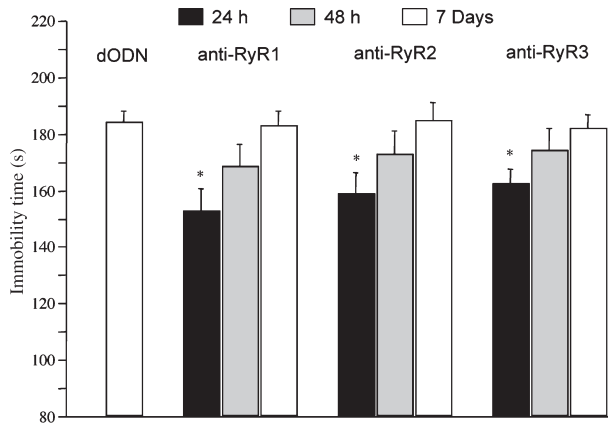

Fig. 4 Time-course of the antidepressant-like effect in RyR1, RyR2, and RyR3 knockdown mice. The immobility time values were recorded 24 h, 48 h, and 7 days after the end of the aODN treatment (5 nmol per mouse). Vertical lines represent SEM; $*p < 0.01$ in comparison with control mice.

i.c.v. treatment, progressively diminished at 24 and 48 h, and disappeared 7 days after the end of treatment as the immobility time values were all comparable with that of the control group (Fig. 4). In these experimental conditions, the animal sensitivity to antidepressants was unchanged as amitriptyline and clomipramine produced a significant reduction of the immobility time (data not shown).

Effect of intracellular calcium modulators on mouse locomotor behavior

Mice pre-treated with anti-RyR1, anti-RyR2, anti-RyR3, and dODN 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 number of falls from the rotating rod, evaluated before and 15, 30, and 45 min after the beginning of the rota-rod test, showed a lack of impairment in the motor coordination of knockdown animals in comparison with dODN group (Fig. 5a).

The spontaneous mobility (Fig. 5b) and exploratory activity (Fig. 5c) of mice were unmodified in knockdown mice in comparison with dODN group. In the same experimental conditions, D-amphetamine, used as positive control, significantly increased both spontaneous mobility [$F(4,66)$ 4.334] and exploratory activity [$F(4,66)$ 4.011] (Fig. 5b and c).

Discussion

The role of central RyR isoforms in the modulation of depressive states was investigated by using pharmacological modulators and knockdown mice. The RyR blockade and the inhibition of the expression of types 1, 2, and 3 RyR induces an antidepressant-like effect of intensity comparable

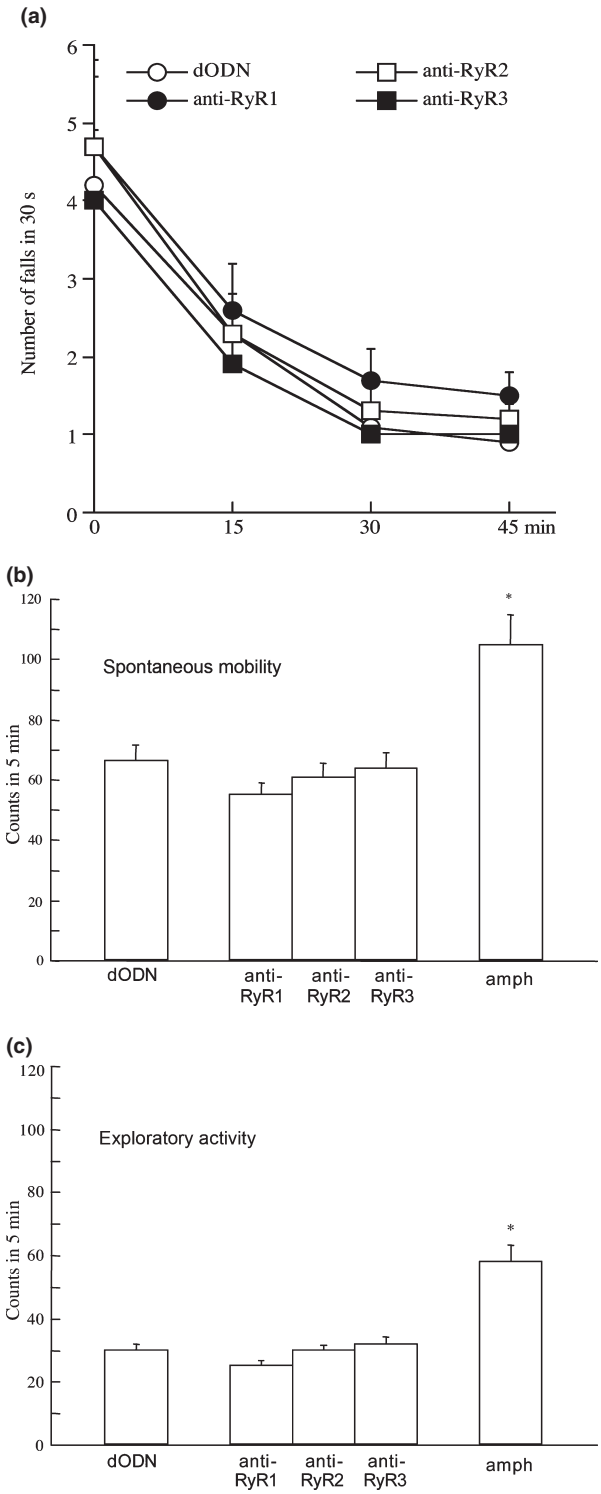

Fig. 5 Lack of influence on mouse motor activity by anti-RyRs. The absence of effect of anti-RyR1, anti-RyR2, and anti-RyR3 on mouse motor coordination (a), spontaneous mobility (b), and exploratory activity (c) was observed in comparison with dODN-treated mice. Amphetamine (amph) was used as positive control. Vertical lines represent SEM; $*p < 0.05$ in comparison with control group.

with the effect produced by widely used antidepressants. Present results represent the first indication of an involvement of RyR subtypes in antidepressant efficacy and help elucidate the physiological role of supraspinal endoplasmic RyRs.

Intracerebroventricular administration of FK506 and ryanodine decreased the immobility time in mice producing an antidepressant-like effect in the mouse forced swimming test. The Ca^{2+} channel-stabilizing proteins calstabin or FK506-binding protein 12 (FKBP12) are a RyR channel stabilizing proteins. Binding of FKBP12 to RyR modulates channel gating by stabilizing the full conductance state and facilitating coupled activation within receptor clusters. Binding of FK506 to FKBP12 as well as depletion of calstabin from RyR increases channel open probability and induces subconductance states, indicating that calstabin is required for stabilizing the closed state of the channel (Foskett *et al.* 2007; Zalk *et al.* 2007). It has been reported that ryanodine blocks Ca^{2+} release from Ca^{2+}/caffeine-sensitive microsomal pools, which are involved in the phenomenon of Ca^{2+}-induced Ca^{2+} release (McPherson *et al.* 1991). Ryanodine reduces the rate at which $[Ca^{2+}]_i$ increase with Ca^{2+} entry (Friel and Tsien 1992). Thus, we can hypothesize that the antidepressant-like response produced by i.c.v. ryanodine might be because of a decrease in $[Ca^{2+}]_i$ at supraspinal level. In confirmation of these data, previous results performed in our laboratory demonstrated that administration of compounds able to increase intracellular Ca^{2+} levels produced a depressant-like effect in mice. By contrast, the treatment with pharmacological compounds able to block Ca^{2+} release from intracellular stores provoked a decrease in mouse immobility time inducing an antidepressant-like effect (Galeotti *et al.* 2006).

Ryanodine is a selective RyR antagonist unable to distinguish among RyR subtypes. To investigate the role played by RyR isoforms, generation of knockdown animals was necessary as 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 because of respiratory failure (Takeshima *et al.* 1995). Similarly to what observed in RyR1 knockout mice, generation of mice carrying a targeted disruption of the RyR2 gene indicate 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 tub (Takeshima *et al.* 1998). These observations ruled out the possibility of using RyR1 and RyR2 knockout mice for behavioral studies. Mice lacking both RyR1 and RyR3 were also generated. These double-mutant mice did not actively move after birth and died, most probably from respiratory failure, as was the case for RyR1-deficient mice (Ikemoto *et al.* 1997).

The capability of the aODN treatment to knockdown RyR protein levels was demonstrated by immunoblotting and immunoprecipitation experiments where the levels of RyR1, RyR2, and RyR3 were determined. A selective decrease in RyR1, RyR2, and RyR3 protein levels was demonstrated in the hippocampus and frontal cortex by using selective antibodies for each RyR isoform. Immunocytochemical studies gave a further characterization of the aODN effect on mouse brain. It was observed that aODN treatment greatly decreases the immunostaining, indication of a selective reduction of RyR protein expression, not only in the cerebellum, area of major presence of RyR isoforms, but also in the mouse frontal cortex and hippocampus.

Types 1, 2, and 3 RyR knockdown mice showed an antidepressant-like behavior indicating the importance of all RyR isoforms in the modulation of a depressive state. Present data shed some light on the physiological role of central RyR subtypes by giving the first indication of their involvement in the induction of an antidepressant-like effect. However, it should be noted that the RyR2 and RyR3 isoforms appear to have a prominent role in comparison with the RyR1 isoform. To obtain a decrease in the immobility time of comparable intensity, a double aODN concentration for anti-RyR1 was necessary. In other words, a significant antidepressant-like effect was induced in anti-RyR2 and anti-RyR3 treated mice with a lower level of inhibition of protein expression in comparison with animals treated with anti-RyR1. This difference could be because of a different role played by the three RyR isoforms in the modulation of a depressive condition. Another possible explanation could be found in the different distribution of RyR isoforms in the brain. Type 2 RyR represents the predominant isoform in the CNS found in widespread brain regions (McPherson and Campbell 1993; Giannini *et al.* 1995). RyR3, the so-called brain isoform, is highly expressed in the CNS as well (Zalk *et al.* 2007). The distribution of the three RyR isoforms in the mouse brain at supraspinal level was also investigated in our study by immunoblotting and immunocytochemistry experiments. ODNs were not delivered selectively in specific brain areas to identify the site of action. However, we might hypothesize a different role of RyR subtypes in antidepressant efficacy on the bases of their distribution in cerebral areas mainly involved in depressive states. RyR2 and RyR3 isoforms were present in microsomes from mouse hippocampus and frontal cortex. Conversely, type 1 RyR has a poor central presence. RyR1 was present in the hippocampus, but it was not detected in mouse cortex, cerebral area of major importance in the modulation of mood. This limited cerebral distribution might justify the lower intensity of the antidepressant-like effect observed in anti-RyR1 treated mice.

Abnormalities of RyRs have been related to the induction of several skeletal-muscle pathologies such as malignant hyperthermia, porcine stress syndrome, and central-core disease (Missiaen *et al.* 2000). Conversely, few data are available on the etiopathological role of RyRs at the CNS level. Deletion of RyR3 results in specific changes in intracellular processes underlying spatial learning and hippocampal synaptic plasticity (Balschun *et al.* 1999). RyR3 knockout mice have impairments of performance in the contextual fear conditioning test, passive avoidance test and Y-maze learning test (Kouzu *et al.* 2000) indicating the importance of RyR3 in the memory and learning processes. Some studies indicate that RyR2, similarly to RyR3, is involved in the modulation of memory processes. An increase in the RyR2 mRNA and protein was found in rats trained in an intensive water maze task (Zhao *et al.* 2000). Present results extend the knowledge of the physiological role of RyR2 and RyR3 at supraspinal level and give the first indication of a central role for RyR1. On the basis of our results, a possible involvement of RyR isoforms in the etiopathology of depressive disorders might be hypothesized.

Antisense ODNs are short synthetic DNA segments complementary to sequences of an mRNA target. aODN specifically binds to targeted mRNA, preventing translation and/or mediating mRNA cleavage by Rnase H and, therefore, transiently inactivating single genes and down-regulating the synthesis of the encoded protein. The reversibility of the aODN effect is fundamental to ensure that the behavioral changes observed were related to the knockdown of the target protein. The antidepressant-like effect induced by anti-RyR1, anti-RyR2, and anti-RyR3 decreased 48 h after the end of the antisense treatment and disappeared 7 days after the last i.c.v. injection. The reversibility of the antidepressant-like effect also indicates a lack of irreversible damage or toxicity on cerebral structures caused by the aODNs. Furthermore, the dODN, used as control ODN treatment, did not modify the immobility time in comparison with naive or saline- and vector-i.c.v. injected mice. The possibility that the antagonism exerted by the aODNs may result from a sequence-independent action on cerebral structures can, therefore, be ruled out.

The aim of this study was to investigate the role of RyR subtypes in the CNS. For this reason, aODNs were administered directly into the cerebral ventricles. Furthermore, intracellular Ca^{2+} plays an important role in the CNS as well as in a variety of peripheral physiological processes. In particular, RyRs are present in the skeletal and cardiac muscle where their activation represents a crucial step for muscle contraction (Rossi and Sorrentino 2002). The i.c.v. administration also avoids the possible appearance of peripheral effects that could lead to a misinterpretation of the results obtained.

The forced swimming test is widely used to predict the antidepressant action of drugs in humans. The majority of antidepressants decreased the immobility time of mice in this test (Petit-Demouliere *et al.* 2005), and their effectiveness correlates significantly with clinical potency (Willner 1984). However, this animal model has also some drawbacks

represented by the possibility to obtain some false positives or negatives (Borsini and Meli 1988; Detke and Lucki 1996). Drugs enhancing motor activity and spontaneous motility may give a 'false' positive effect whereas compounds inducing ataxia may give a 'false' negative effect. It is suitable to check the induction of any alteration in the mouse motor activity, in parallel with forced swimming test. aODN treatment was devoid of any visible alteration of animals' behavior. Additional behavioral tests were also conducted to unmask an altered motor coordination (rota-rod test), spontaneous motility, and exploratory activity (hole-board test) induced by RyR knockdown. Any influence on motor activity was excluded. These results were of particular relevance as it has been observed that deletion of RyR3 induces an increased speed of locomotion and a mild tendency to circular running (Balschun *et al.* 1999). Present results indicate that a gene-specific cerebral RyR isoform level reduction by using an antisense strategy is compatible with animal life, devoid of behavioral side effects, and represents a useful tool to investigate physiological and/or pathological role of RyR-mediated cellular events.

We conclude that cerebral reduction of the expression of types 1, 2, and 3 RyR induces an antidepressant-like effect in mice. These findings identify cerebral RyR isoforms as critical targets underlying depressive states and should facilitate the comprehension of the etiopathology of mood disorders and help developing new therapeutical strategies.

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