An antidepressant behaviour in mice carrying a gene-specific InsP3R1, InsP3R2 and InsP3R3 protein knockdown

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ABSTRACT

Evidence has accumulated for the involvement of Ca^{2+} in the pathophysiology of mood disorders. Elevations in both resting and stimulated intracellular Ca^{2+} levels in patients with affective disorders have been reported. The role of inositol-1,4,5-trisphosphate receptors (InsP3Rs), which allow mobilization of intracellular Ca^{2+} stores, was, then, investigated in the mouse forced swimming test. InsP3R antagonists (heparin, xestospongin C) as well as an inositol monophosphatase inhibitor (LiCl) showed an antidepressant activity of intensity comparable to clinically used antidepressants. InsP3Rl, InsP3R2 and InsP3R3 knockdown mice were obtained to investigate the role of InsP3R isoforms. We generated mice carrying a cerebral knockdown of InsP3Rl, InsP3R2 and InsP3R3 proteins by administering antisense oligonucleotides complementary to the sequence of InsP3Rl, InsP3R2 and InsP3R3. These antisense-treated mice showed a specific InsP3R protein level reduction in the mouse cerebral cortex and hippocampus, demonstrated by immunoblotting, immunoprecipition and immunocytochemistry experiments. Knockdown mice for each InsP3R isoforms showed an antidepressant behaviour and the induced phenotype was reversible disappearing 7 days after the end of the treatment. The absence of impairment of locomotor activity and spontaneous mobility in InsP3R knockdown mice was revealed. These results indicate the involvement of the InsP3R-mediated pathway in the modulation of depressive conditions and may be useful for the development of new therapeutical strategies for the treatment of mood disorders.

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1. Introduction

Calcium is a ubiquitous intracellular signalling molecule that is required for initiating and regulating a wide range of neuronal functions, including neurotransmitter release, synaptic plasticity, neurite outgrowth, and neurodegeneration (Berridge, 1998; Ciccolini et al., 2003). 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 (InsP3R) and the ryanodine receptor (RyR): InsP3R is a key molecule for InsP3-induced Ca^{2+} release, whereas RyR is important for Ca^{2+}-induced Ca^{2+} release (Foskett et al., 2007; Zalk et al., 2007). Considering the importance of calcium signals for cellular functions, it is not surprising that functional abnormalities in endoplasmic calcium channels are also involved in pathological conditions that are related to disturbance in calcium homeostasis. Several skeletal-muscle pathologies such as malignant hyperthermia, porcine stress syndrome, central-core disease, can be caused by mutations in ryanodine receptors (Missiaen et al., 2000). Brain InsP3Rs have been hypothesized to contribute to the pathology of episthotonos in mice (Street et al., 1997). Disturbance in InsP3R and RyR appears to be involved in neurodegenerative diseases such as Alzheimer's disease (Mattson and Chan, 2003).

Evidence has accumulated for the involvement of Ca^{2+} also in the pathophysiology of mood disorders. Platelet intracellular calcium mobilization stimulated by serotonin is enhanced in depressed patients (bipolar, major, melancholic) in comparison with normal subjects (Kusumi et al., 1991; Mikuni et al., 1992; Eckert et al., 1993; Tomiyoshi et al., 1999). In platelets from unmedicated bipolar patients, the increase in intracellular calcium after thrombin stimulation is significantly higher than that measured in healthy controls (Kusumi et al., 1992; Suzuki et al., 2001). Some investigations also suggested that baseline

intracellular calcium levels are elevated in platelets or lympho-blasts of patients with bipolar affective disorders (Dubovsky et al., 1994; Emamghoreishi et al., 1997). Understanding the basis for this dysregulation of calcium homeostasis will be crucial for under-standing the pathogenesis of affective disorders.

Even if elevations in both resting and stimulated intracellular Ca^{2+} levels in patients with affective disorders have been reported, little is known on the involvement of the intracellular Ca^{2+}-release channels in mood disorders. Three distinct receptors recognise InsP3, named InsP3Rl, InsP3R2 and insP3R3. InsP3Rs are recognized as a protein family of tetrameric ligand-gated Ca^{2+} channels, which allow mobilization of intracellular Ca^{2+} stores in response to acti-vation of cell surface receptors linked to InsP3 generation (Berridge, 1993). InsP3R monomers are large proteins with a calculated molecular mass of about 300 kDa and all three isoforms of InsP3R share 60–70% amino acid similarity (Taylor et al., 1999). The aim of this study was to determine the role of the three InsP3R isoforms in a depressive task by using the mouse forced swimming test. To this purpose we inhibited the expression of each InsP3 receptor subtype by using antisense oligonucleotides targeting InsP3R mRNA sequences, which are unique in the mouse genome, and therefore assure stringent target selectivity.

2. Experimental procedures

2.1. Antisense oligonucleotides

Phosphodiester oligonucleotides (ODNs) protected by terminal phosphor-othioate double substitution (capped ODNs) against exonuclease-mediated degra-dation were purchased from Tib-Molbiol (Genoa, Italy). The sequences are the following: *anti-IP3Rl*: 5′-C*T*C GAC ATT TTG TCA GAC* A*T-3′, targeting the mRNA (Genbank NM_010585.2) at positions 329–348; *anti-IP3R2*: 5′-A*G*G AAG CTG GAC ATT TTG* T*C-3′ targeting the mRNA (Genbank NM_019923.3) at positions 41–60; *anti-IP3R3*: 5′-C*T*G GAC ATT TCA TTC ATG* G*C-3′ targeting the mRNA (Genbank NM_080553.2) at positions 48–67 (* indicates phosphorothioate residues). A 20-mer fully degenerated ODN (dODN) 5′-N*N*N NNN NNN NNN NNN NNN* 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, Italy) 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 5 μl solution on days 1, 2 and 3. Behavioural tests were performed on day 4, 24 h after the last i.c.v. injection of ODNs.

2.2. Preparation of membranes

Mouse brains were dissected to separate specific areas. Mouse cerebellum, hippocampus, cortex and striatum were homogenized in an homogenization buffer. The homogenate was centrifuged at $9000 \times g$ for 15 min at 4 °C and the low speed pellet was discarded. The microsomal membranes were obtained from the super-natant of the $9000 \times g$ spin by centrifugation at $100,000 \times g$ 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 RadLaboratories, Milan, Italy).

2.3. Immunoblot analysis

Membrane homogenates (30 μg) of control and antisense-treated mice were separated on 6% SDS-PAGE and transferred onto nitrocellulose membranes (60 min at 100 V) using standard procedures. Membrane was blocked in PBST (PBS con-taining 0.1% Tween) containing 5% non-fat dry milk for 90 min. Following washings, blots were incubated overnight at 4 °C with specific antibodies (SantaCruz Biotechnology, CA, USA) against InsP3Rl, InsP3R2 or InsP3R3 (1:1000 dilution). After being washed with PBST, the nitrocellulose membrane was incubated with rabbit anti-goat 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 were standardized for all the blots. Densitometric analysis of scanned images was per-formed on a Macintosh iMac computer using the public domain NIH Image program. Measurements in control samples were assigned a relative value of 100%.

2.4. Immunoprecipitation of InsP3Rl, InsP3R2 and InsP3R3

Immunoprecipitation was carried out on 1 ml of microsomal fraction containing 100 μg proteins by incubation for 2 h at 4 °C with 10 μg of specific antibodies against InsP3Rl, InsP3R2 or InsP3R3. 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 $1000 \times g$ for 5 min at 4 °C, washed three times with homogenization buffer. Pellets were finally resuspended in 40 μl elec-trophoresis sample buffer, boiled for 5 min, and samples were processed by SDS-PAGE.

2.5. Tissue processing and immunocytochemistry InsP3Rs

Mice were anesthetized with chloral hydrate 12% and perfused with para-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. Afterpost-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 LEICA CM1800. 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 room temperature. 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-InsP3Rl, anti-InsP3R2 or anti-InsP3R3 (1:100) (Santa Cruz Biotechnology Inc., Santa Cruz, CA), or anti-RyR2 (kind gift of Prof. Sorrentino, University of Siena, Italy). After rinsing in 0.1% Triton PBS, sections were incubated for 1 h at RT with diluted biotinylated secondary antibody solution (1:200). The6 sections were stained using the avidin–biotin–peroxidase complex (ABC) method with a Vectastain Kit (Vector Laboratories Inc., Burlingame, CA) and then washed in PBS before development with DAB method (Vector Laboratories Inc., Burlingame, CA). 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).

2.6. Animals

Male Swiss albino mice (23–25 g) 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 a.m. Animals were naive and used only once. 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.

2.7. Intracerebroventricular injection technique

Intracerebroventricular (i.c.v.) administration was performed under ether anesthesia, as previously described (Galeotti et al., 2003). During anaesthesia, 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. Injec-tions 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.

Drug concentrations were prepared so that the necessary dose could be administered in a volume of 5 μl per mouse.

2.8. Forced swimming test

The forced swimming test used was the same as described by Porsolt et al. (1977). Briefly, mice were dropped individually into glass cylinders (height: 25 cm, 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. Twelve to fifteen mice per group were tested.

2.9. 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 × 4 in an equidistant, grid-like manner. Mice were placed on the centre 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 signalled 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. Twelve to fifteen mice per group were tested.

2.10. 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 3 and more than 6 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. Twelve to fifteen mice per group were tested.

2.11. Drugs

The following drugs were used: heparin sodium salt (mol. wt: approx. 6000), clomipramine hydrochloride, amitriptyline hydrochloride, lithium chloride (Sigma, Milan, Italy), xestospongin C, D-myo-inositol 1,4,5-trisphosphate hexasodium salt, L-myo-inositol 1,4,5-trisphosphate hexapotassium salt (Calbiochem, Milan Italy). Other chemicals were of the highest quality commercially available. Xestospongin C was dissolved in 0.01% ethanol whereas all other drugs were dissolved in isotonic (NaCl 0.9%) saline solution immediately before use. Drag concentrations were prepared so that the necessary dose could be administered in a volume of 5 μl per mouse by intracerebroventricular (i.c.v.) injection and 10 ml kg^{-1} by subcutaneous (s.c.) injection.

2.12. Drug administration

LiCl (0.4–400 mg kg^{-1} s.c.) was administered 18 h before the forced swimming test, heparin (0.08–6.6 nmol per mouse i.c.v.), xestospongin C (1–100 pmol per mouse i.c.v.), D-myo-inositol (5 pmol per mouse i.c.v.) and L-myo-inositol (5 pmol per mouse i.c.v.) were injected 15 min before behavioural test. Clomipramine (25 mg kg^{-1} s.c.) and amitriptyline (15 mg kg^{-1} s.c.) were injected 30 min before the test. D-amphetamine (2 μg per mouse i.c.v.) was administered 15 min before the hole board test. Doses and administration schedule were chosen on the basis of time-course and dose–response experiments previously performed in our laboratory ODNs were i.c.v. injected once daily on days 1, 2 and 3. Behavioral tests were performed on day 4, 24 h after the last i.c.v. injection of ODNs. To perform ODN time-course experiments, the immobility time values were recorded on day 5 and 10 corresponding to 36 h and 7 days after the end of the ODN treatment, respectively. In time-course, the hole board and rota rod tests' ODNs were injected at doses of 0.5 nmol per mouse (anti-InsP3Rl and anti-InsP3R3), or 1 nmol per mouse (anti-InsP3R2).

Brain areas to conduct western blotting and immunocytochemistry experiments were collected on day 4, 24 h after the last i.c.v. injection of ODN at doses of 0.5 nmol per mouse (anti-InsP3Rl and anti-InsP3R3), or 1 nmol per mouse (anti-InsP3R2), and on day 11.

2.13. Statistical analysis

All experimental results are given as the mean + S.E.M. An analysis of variance ANOVA, followed by Fisher's post hoc comparison, was used to verify significance between two means of behavioural results. P values of less than 0.05 were considered significant.

3. Results

3.1. Antidepressant-like effect of pharmacological modulators of InsP3R pathway

The administration of the InsP3 receptors antagonist heparin (0.08–6.6 nmol per mouse i.c.v.) produced a dose-dependent antidepressant-like effect (Fig. 1, panel A). The reduction of immobility time produced by heparin (6.6 nmol per mouse i.c.v.) was reversed by D-myo inositol (5 pmol per mouse i.c.v.) whereas it remained unchanged by pre-treatment with L-myo-inositol (5 pmol per mouse i.c.v.), used as negative control (Fig. 1, panel A). D-myo inositol (5 pmol per mouse i.c.v.) and L-myo inositol (5 pmol per mouse i.c.v.), when administered alone, did not modify the mouse immobility time in comparison with control group (Fig. 1, panel A).

Similarly to heparin, xestospongin C (1–100 pmol per mouse i.c.v.), antagonist of InsP3 receptors, showed antidepressant-like properties (Fig. 1, panel B). LiCl, an inositol monophosphatase inhibitor, produced an antidepressant-like effect in a dose-dependent manner (0.4–400 mg kg^{-1} s.c.) (Fig. 1, panel C). The decrease of the immobility time produced by heparin, xestospongin C and LiCl

was comparable to that exerted by clomipramine (25 mg kg^{-1} s.c.) used as reference drug (Fig. 1).

3.2. Cerebral InsP3Rl, InsP3R2 and InsP3R3 protein knockdown induced by antisense treatment

The level of expression of InsP3Rl, InsP3R2 and InsP3R3 in the cerebellum, cortex and hippocampus of knockdown animals was detected in comparison with mice treated with dODN, used as control ODN. Fig. 2 shows a representative immunoblot where a prominent approximately 260 kDa protein band was observed in the cortex (panel A) and the hippocampus (panel B), indicating that all three receptor subtypes were expressed in the above-mentioned cerebral areas. A statistically significant reduction of the expression of InsP3Rl, InsP3R2 and InsP3R3 after aODN treatment, in comparison with mice treated with the corresponding dODN, was observed in terms of detected protein levels in the cortex (Fig. 2a) as well as in the hippocampus (Fig. 2b). Densitometric analysis of all samples revealed that every aODN treatment decreased expression of the corresponding receptors in both cerebral areas investigated. A comparable inhibition of protein expression was obtained in the cerebellum, area where InsP3Rs are abundantly expressed and, for this reason, used as positive control (data not shown). Levels of expression of InsP3Rl, InsP3R2 and InsP3R3 proteins returned to normal 7 days after the end of the aODN treatment (data not shown). Immunoprecipitation experiments also produced similar results in both cortex and hippocampus (data not shown).

The dODN did not significantly change the level of immunostaining when compared with that of naive animals. 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 InsP3Rl, InsP3R2 and InsP3R3 down-regulated brain region (Fig. 2a and b). Cross-reactivity of the primary antibodies used was excluded.

Brain slices of treated and control mice were immunostained to show the localization and level of expression of InsP3Rs. Results obtained in anti-InsP3Rl knockdown animals, taken as example, were reported in Fig. 3. In the cerebellum of dODN-treated mice InsP3Rl was highly expressed and concentrated in Purkinje cells (Fig. 3a). A lower immunostaining was observed in anti-InsP3Rl treated mice (Fig. 3b). This protein was also detected in the mouse hippocampus (Fig. 3c) and, similarly to cerebellum, a reduction of protein expression was observed in InsP3Rl knockdown animals (Fig. 3d). RyR2 protein was used as positive control protein and no difference in the protein expression was observed between control and InsP3Rl knockdown mice (data not shown).

3.3. Antidepressant-like effect in InsP3Rl, InsP3R2 and InsP3R3 knockdown mice

The effect produced by a reduction of InsP3R protein levels was evaluated in the mouse forced swimming test. Repeated administration of anti-InsP3Rl induced an antidepressant-like effect in a dose-dependent manner (0.1–5 nmol per mouse). A bell-shaped curve was obtained (Fig. 4a). Similarly to anti-InsP3Rl, the administration of anti-InsP3R2 (Fig. 4b) and anti-InsP3R3 (Fig. 4c) produced a dose-dependent antidepressant-like effect (0.1–3 nmol per mouse i.c.v.). The i.c.v. administration of ODNs did not modify the animal response in the forced swimming test. The immobility time values recorded in the dODN-treated group were comparable to those of the naive, saline-treated and vector-treated (DOTAP 13 μM) mice (Fig. 4d). In these experimental conditions the administration of amitriptyline (15 mg kg^{-1} s.c.) and clomipramine (25 mg kg^{-1} s.c.), used as reference compounds, produced a statistically significant antidepressant-like effect (Fig. 4d). The decrease
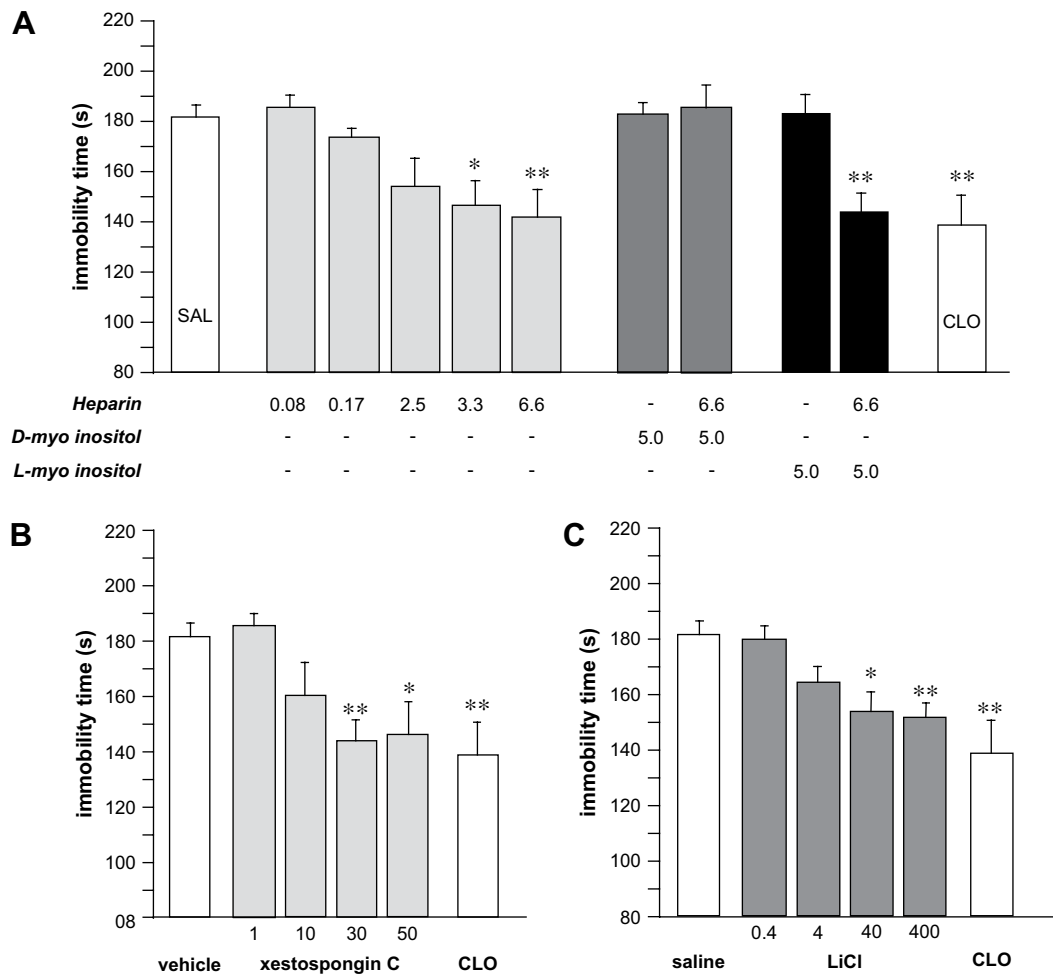
Fig. 1. Antidepressant-like effect of InsP3R pharmacological blockers. Antidepressant-like effect induced by heparin (panel A), xestospongin C (panel B) and LiCl (panel C) in the mouse forced swimming test. Doses of heparin (nmol per mouse i.c.v.), xestospongin C, D-myo inositol, L-myo inositol (pmol per mouse i.c.v.) and LiCl (mg kg^{-1} s.c.) were reported under each column; CLO: clomipramine 25 mg kg^{-1} s.c.; vehicle: 0.01% ethanol; vertical lines represent SEM; between 12 and 15 mice were tested. $^*P < 0.05$, $^{**}P < 0.01$ in comparison with saline-treated mice.

of the immobility time induced by anti-InsP3Rl, anti-InsP3R2 and anti-InsP3R3 was of intensity comparable to that induced by the antidepressant drugs amitriptyline and clomipramine (Fig. 4d).

To evaluate the reversibility of the aODN effects, mice were treated with the aODNs on day 1, 2 and 3; 7 days after the last i.c.v. injection, mice were killed and the cortex and hippocampus were dissected and examined for the protein levels of InsP3Rl, InsP3R2 and InsP3R3 in comparison with mice treated with dODN. Fig. 5a shows a representative immunoblot where three distinct prominent protein bands of approximately 26 kDa, corresponding to InsP3Rl, InsP3R2 and InsP3R3, were observed in the mouse hippocampus. InsP3R subtypes protein expression in aODN treated mice was similar to control animals. Time course experiments showed that the antidepressant-like effect produced by the highest effective doses of anti-InsP3Rl (0.5 nmol per mouse), anti-InsP3R2 (1 nmol per mouse) and anti-InsP3R3 (0.5 nmol per mouse), slowly diminished 36 h after the end of repeated treatment and disappeared after 7 days (Fig. 5b).
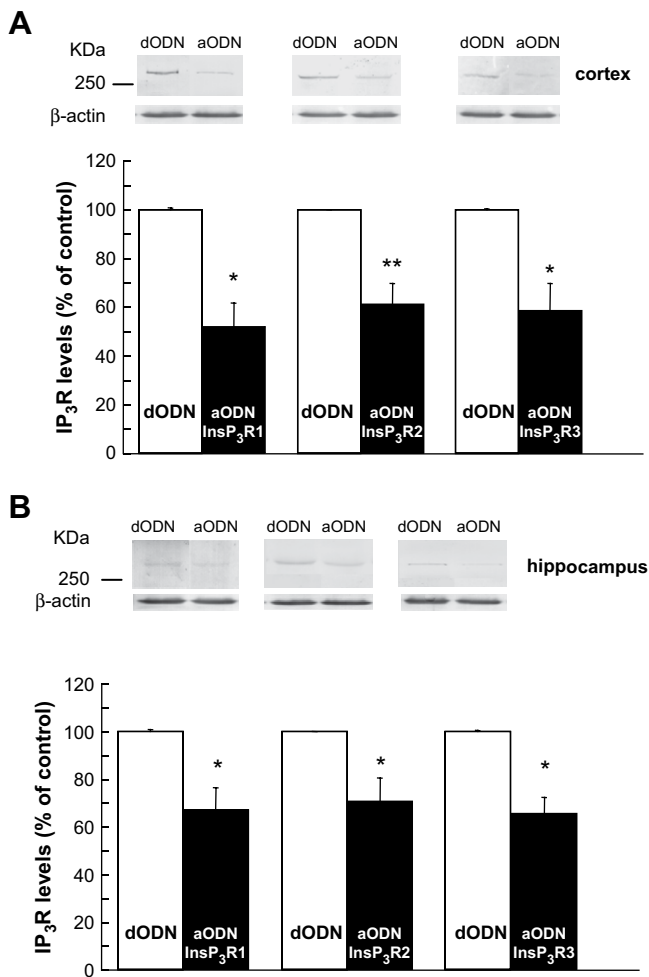
3.4. Effect of InsP3Rl, InsP3R2 and InsP3R3 knockdown on mouse locomotor behaviour

Repeated administration of anti-InsP3Rl neither produced behavioural side effect such as tremors, convulsions, etc. nor induced lethality up to the dose of 5 nmol per mouse. By contrast,

anti-InsP3R2 and anti-InsP3R3 at the dose of 5 nmol per mouse produced convulsions and death in 90% and 80% of animals, respectively. The dose of 3 nmol was endowed with a lower toxicity (62.9–68.5% of lethality) and survived animals showed neither tremors nor convulsions. Treatment with lower doses did not induce any lethality. The aODNs were tested in order to unmask their potential effect on mouse motor behaviour. The spontaneous mobility (Fig. 6a) and the exploratory activity (Fig. 6b) of mice were unmodified by pretreatment with aODNs in comparison with the dODN group. D-amphetamine (2 mg kg^{-1} s.c.), used as positive control, significantly increased both parameters evaluated (Fig. 6a and b). The lack of any impairment in the motor coordination of animals pretreated with aODNs in comparison with the dODN group was demonstrated (Fig. 6c). The highest effective doses of heparin and xestospongin C did not modify mouse spontaneous mobility, exploratory activity and motor coordination (data not shown).

4. Discussion

Our experiments show that InsP3R-mediated pathway involved the modulation of depressive states in mice. Pharmacological blockade of InsP3Rs activity as well as a gene-specific cerebral InsP3Rl, InsP3R2 and InsP3R3 protein knockdown is endowed with an antidepressant behaviour of intensity comparable to that exhibited by clinically used antidepressant drugs.

Fig. 2. Cortical and hippocampal InsP3R protein levels in knockdown animals. Reduction of InsP3Rl, InsP3R2 and InsP3R3 protein expression in the mouse cortex (panel A) and hippocampus (panel B) by aODN treatment in comparison with corresponding dODN-treated mice. Samples (30 μg protein/lane) of microsomal fractions of mouse cortex and hippocampus were resolved on 6% SDS-PAGE, transferred to nitrocellulose and probed with an InsP3Rl, InsP3R2 or InsP3R3 antibody. Inside each panel is the densitometric quantitation of immunoreactive protein expressed relative to control. Data are expressed as mean ± SEM of band intensities from each of the four groups ($n = 7$ per group). $^{*}P < 0.05$ versus control.

The antidepressant-like properties of the InsP3R pharmacological antagonists heparin (Jonas et al., 1997) and xestospongin C (Gafni et al., 1997) were demonstrated by present results. I.c.v. administration of both the membrane permeable low molecular weight heparin (Jonas et al., 1997) and xestospongin C produced a decrease of the immobility time in the mouse forced swimming test. This effect was reversed by D-myo inositol, compound which generates InsP3, whereas L-myo inositol, used as negative control, did not produce any effect. These data demonstrate that the heparin and xestospongin C antidepressant activity was related to a blockade of cerebral InsP3Rs. An indication of a positive effect of heparin in humans as well was reported. The complete disappearance of depressive symptoms after addition of subcutaneous calcium heparin to fluoxetine was observed in a patient suffering from a recurrent depression resistant to conventional antidepressant therapy (Maluquer et al., 2002). On the bases of our findings, we can hypothesize that the beneficial effect of heparin might be a consequence of its antidepressant activity.

To further elucidate the InsP3-mediated pathway in the induction of an antidepressant behaviour, LiCl, an uncompetitive inhibitor of inositol monophosphatase, which regenerates inositol from inositol monophosphate, was used. The LiCl-induced inhibition depletes inositol and prevents the formation of InsP3 (Phiel and Klein, 2001). LiCl pretreatment decreases the immobility time indicating that the inhibition of the synthesis of InsP3 is necessary to counteract the induction of a depressant condition. In support to our findings, it has been reported that NCS-1, a high affinity, low capacity, calcium binding protein, modulates calcium signalling by enhancing InsP3-mediated activity of InsP3Rl. The association NCS-l/InsP3Rl was attenuated by lithium (Schlecker et al., 2006).

Three different InsP3R isoforms are expressed in the central nervous system. To further elucidate the involvement of the InsP3R-mediated pathway in mood disorders, the role of types 1–3 InsP3Rs was investigated in a depressant task. InsP3Rl, InsP3R2 and InsP3R3 knockdown animals showed an antidepressant-like behaviour indicating the importance of all three InsP3R isoforms in the modulation of a depressive state. Present results further elucidate the physiological role of supraspinal endoplasmic InsP3Rs and give indication for the involvement of each InsP3R isoforms in the pathological mechanism of mood disorders. In agreement with present data, several studies have shown an overstimulated phosphoinositide signalling system in affective disorders. The majority of clinical studies available have been conducted in peripheral blood elements since the calcium mobilization studies require fresh, viable cell preparations, which make postmortem samples not always suitable. In particular, platelets of affected people are often used as models for neurons because they have similar signalling features and it is possible to obtain samples from affected individuals. Results from depressed patients evidenced the existence of a greater sensitivity of inositol–phospholipid second messenger system (Bohus et al., 1996) and an altered 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 InsP3 binding sites and expressed InsP3 receptor protein levels have been noted in platelets from depressed patients (Dwivedi et al., 1998; Rosel et al., 2000).

Among the three InsP3R isoforms, InsP3Rl appears to have a prominent role. InsP3Rl knockdown animals showed a decrease of immobility time of greater intensity than InsP3R2 and InsP3R3 knockdown mice. To obtain an immobility time decrease of comparable intensity, a double anti-InsP3R2 and anti-InsP3R3 aODN concentration must be used. These data indicate that a lower level of InsP3Rl protein expression inhibition is sufficient to obtain a phenotype resistant to the induction of a depressive state. To further support our results suggesting the importance of type 1 InsP3R in mood disorders, a reduction of InsP3Rl mRNA in rat brain was observed after a single electroconvulsive shock (Kim et al., 2001).

Type 1 InsP3R is the major neuronal type of InsP3R in the central nervous system, predominantly concentrated in cerebellar Purkinje cells, cerebral cortex, CA1 region of hippocampus, basal ganglia and thalamus (Mikoshiba, 1997; Ross et al., 1992). It is not surprising that an altered InsP3Rl functionality at the central nervous system level may be associated with pathological conditions. Loss of InsP3Rl in mice is associated with ataxia and seizures (Matsumoto et al., 1996), as well as a deficient cerebella long-term depression (Matsumoto and Nagata, 1999). A several-fold reduction of immunostained InsP3Rl was found in two cerebella obtained from patients suffering from ataxia (Zecevic et al., 1999), indicating that the above-mentioned findings on mice may apply to some forms of ataxia in humans as well. In several neurodegenerative diseases, such as Alzheimer disease and Huntington disease, and in brain ischemia, InsP3Rl appears to be selectively down-regulated (Zhang et al., 1995; Tang et al., 2003; Haug et al., 1996; Kasri et al., 2004). We now postulate the possible involvement of the InsP3Rl subtype in depressive disorders.

control
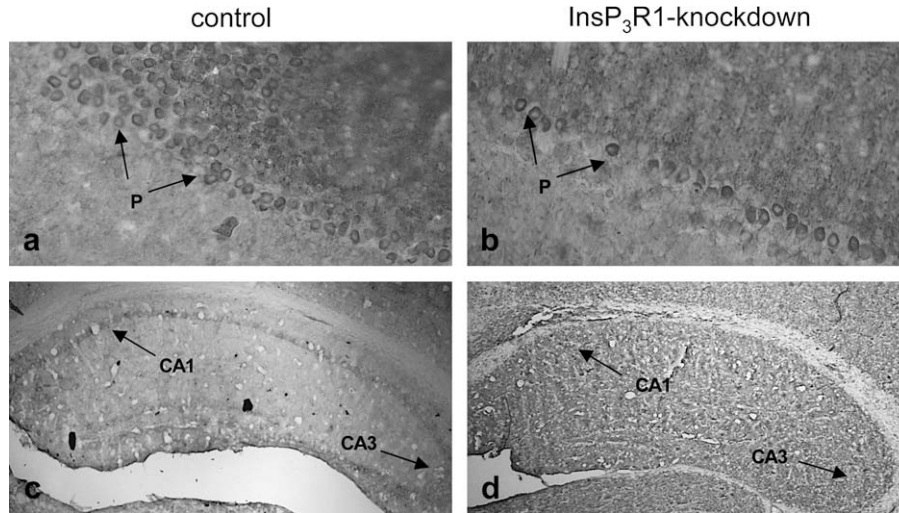
InsP₃R1-knockdown

Fig. 3. Distribution and levels of InsP3Rl protein in InsP3Rl knockdown animals. Reduction of InsP3Rl protein expression in the mouse brain of InsP3Rl knockdown mice. Representative photomicrographs for InsP3Rl immunoreactivity in cerebellar Purkinje cells body (P) (panels A and B) and hippocampal slices (panels C and D).

An antidepressant-like behaviour was also observed in InsP3R2 and InsP3R3 knockdown mice. Although the type 2 InsP3R is present in many tissues, high levels are found in spinal cord and glial cells (Sharp et al., 1999). At the subcellular level, it was found in the cell body but was excluded from the nuclear region (Sheppard et al., 1997; Simpson et al., 1997). Type 3 InsP3R is found in the kidney, gastrointestinal tract, pancreatic islets (Taylor et al., 1999; Patel et al., 1999; Nathanson et al., 1994). Loss of InsP3R3 is found in humans with bile duct obstruction (Shibao et al., 2003). Exocrine dysfunction of the salivary glands and pancreas was observed in InsP3R2 and InsP3R3 double knock-out mice (Futatsugi et al., 2005). These results indicate the importance of InsP3R3 at peripheral level. However, it is also detected in some neurons,

especially in neuron terminals (Sharp et al., 1999). The physiological role of these two receptor subtypes in the central nervous system is not clearly elucidated. Present results provide the first evidence for an involvement of types 2 and 3 InsP3R in the mechanism underlying the induction of a depressive condition.

The specific gene expression knockdown of each of the three InsP3R subtypes was obtained by an oligonucleotide antisense strategy. To investigate the role played by InsP3Rs at the central nervous system level, the aODNs against the types 1–3 InsP3R were administered directly into the mouse cerebral ventricles in order to avoid the appearance of peripheral effects. aODNs are short synthetic DNA segments complementary to sequences of an mRNA target. aODN specifically binds to targeted mRNA, preventing
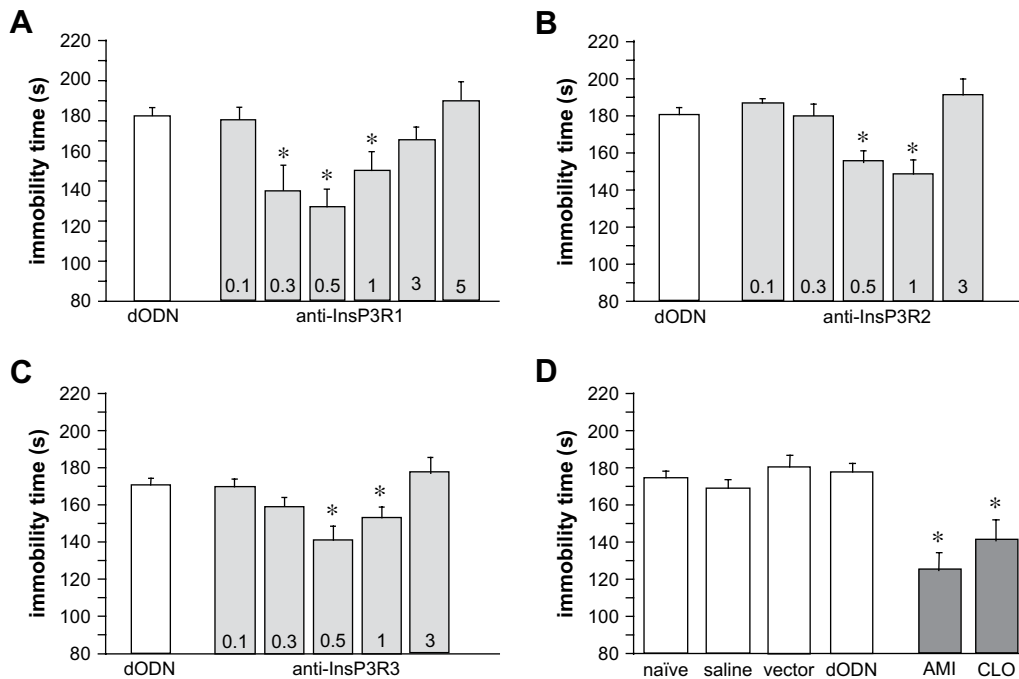
Fig. 4. Antidepressant-like behaviour in InsP3Rl, InsP3R2 and InsP3R3 knockdown mice. Reduction by anti-InsP3Rl (A), anti-InsP3R2 (B) and anti-InsP3R3 (C) of the immobility time was detected in the mouse forced swimming test in comparison with amitriptyline (15 mg kg^{-1} s.c.) and clomipramine (25 mg kg^{-1} s.c.), used as reference drugs. (D) Lack of effect on immobility time in the dODN-treated group in comparison with naïve, saline-treated and vector-treated (DOTAP 13 µM) mice. aODN doses administered (nmol per mouse i.c.v.) were reported in each column. Vertical lines represent SEM; between 12 and 15 mice were tested. $^{*}P < 0.05$, $^{**}P < 0.01$ in comparison with control mice.
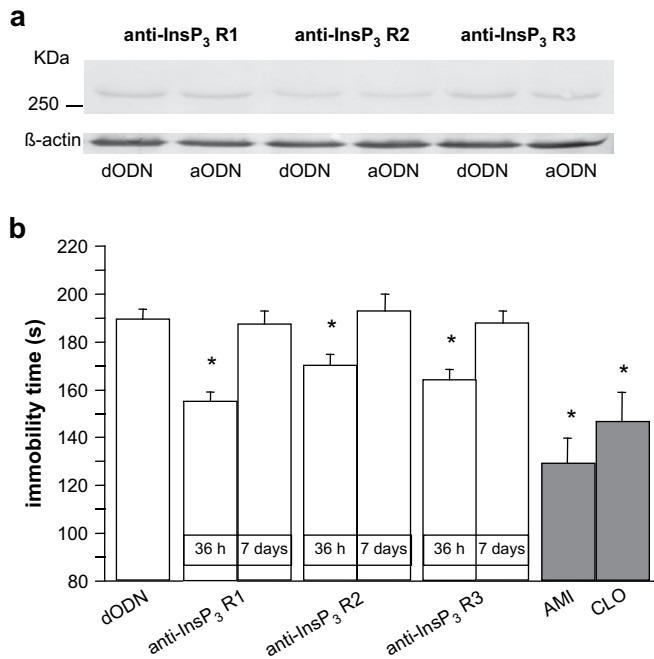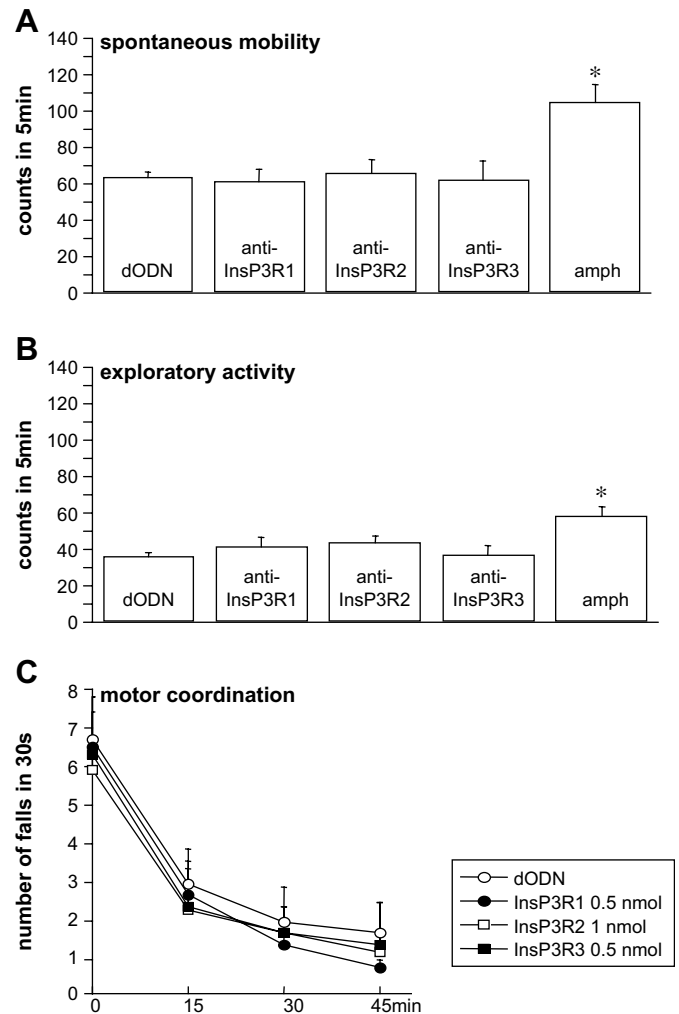
Fig. 5. Reversibility of InsP3Rl, InsP3R2 and InsP3R3 protein knockdown and antidepressant behaviour in aODN-treated mice in comparison with corresponding dODN-treated mice. (A) Samples (30 μg protein/lane) of microsomal fractions of mouse hippocampus were resolved on 6% SDS-PAGE, transferred to nitrocellulose and probed with InsP3Rl, InsP3R2 and InsP3R3 antibody. (B) The immobility time values were recorded 36 h and 7 days after the end of the treatment with anti-InsP3Rl (0.5 nmol per mouse), anti-InsP3R2 (1 nmol per mouse) and anti-InsP3R3 (0.5 nmol per mouse). Vertical lines represent SEM; between 12 and 15 mice were tested. $*P < 0.01$ in comparison with control mice.

Fig. 6. Lack of induction of motor side effects in InsP3R knockdown mice. Lack of alteration on spontaneous mobility (panel A), exploratory activity (panel B) and motor coordination (panel C) in InsP3Rl-, InsP3R2- and InsP3R3-knockdown mice, in comparison with dODN-treated mice. The test was performed 24 h after the end of the treatment with anti-InsP3Rl (0.5 nmol per mouse), anti-InsP3R2 (1 nmol per mouse) and anti-InsP3R3 (0.5 nmol per mouse). Amph: amphetamine 2 mg kg^{-1} s.c.; vertical lines represent SEM. $*P < 0.05$ versus control group.

translation and/or mediating mRNA cleavage by RNase H and, therefore, transiently inactivating single genes and down-regulating the synthesis of the encoded protein. Immunoblotting, immunoprecipitation and immunocytochemistry experiments illustrated a selective decrease of InsP3Rl, InsP3R2 and InsP3R3 protein levels in the cerebellum, hippocampus, and cortex of animals treated with the corresponding aODN in comparison with the degenerated oligonucleotide (dODN)-treated mice. These results showed that the antidepressant phenotypic effect was related to a selective knockdown of the InsP3R isoforms. Furthermore, the dODN, used as control for the unspecific effects attributable to the administration of nucleic acids irrespective of their specific sequence, did not modify the immobility time in comparison with naive or saline- and vector-i.c.v. injected mice. This observation ruled out the possibility that the antidepressant behaviour showed by InsP3R knockdown animals may have resulted from a sequence-independent action on cerebral structures. The immunoblotting results are in agreement with recent data obtained in our laboratory which demonstrated a widespread cerebral reduction of InsP3R subtypes in knockdown mice (Galeotti et al., 2007). Present results showed that a cortical and hippocampal selective reduction of InsP3R isoforms can be obtained with a aODN concentration at least three times lower than that previously used by us.

The antidepressant-like effect induced by anti-InsP3Rl, anti-InsP3R2 and anti-InsP3R3 decreased 36 h after the end of the aODN treatment and disappeared after 7 days. The reversibility of the induced phenotype is a requirement for a proper and correct functionality of aODNs. It indicates a lack of irreversible damage or toxicity on cerebral structures as well.

aODN-treatment produced a bell-shaped curve of modulation of the immobility time. This biphasic effect was similar to the effect produced by the physiological modulators of InsP3Rs (Iino, 1990; Finch et al., 1991).

The forced swimming test is widely used to predict the antidepressant action of drugs in humans. In this test, the majority of antidepressants decreased the immobility time of mice and their effectiveness correlates significantly with clinical potency (Petit-Demouliere et al., 2005). However, this animal model has also some drawbacks represented by the possibility to obtain some false positives or negatives by drugs that modify motor activity, which, therefore, may alter the immobility time values recorded. aODN behavioural effects were tested before the forced swimming test was performed, to make sure that treatments did not alter the normal locomotor activity of the mice. An influence of the substances used on spontaneous motility 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 effective doses was excluded. The results of the rota rod and hole-board tests were of particular relevance since it has been observed that mice lacking

type 1 InsP3R showed severe ataxia (Matsumoto et al., 1996) and motor discoordination (Ogura et al., 2001). This discrepancy can be explained by taking into account that, conversely to the knockout strategy, the antisense strategy permits to modulate the degree of inhibition of the target protein expression. A phenotypic effect devoid of severe toxicity can be obtained. Moreover, compensatory effects taking place during mice development, which can mask important phenotypic effects, are avoided. It should also be noted that higher doses of anti-InsP3R could not be investigated since they induced signs of toxicity (tremors, convulsions etc.) and/or death in animals. The induction of toxicity can also be considered as an indication not only of the diffusion of these compounds in the brain, but also of the consequent reaching of key targets.

Seen as a whole, present results indicate the involvement of the InsP3R-mediated pathway in the modulation of depressive states. In particular, cerebral reduction of types 1–3 InsP3R expression induces an antidepressant-like effect in mice, identifying cerebral InsP3R isoforms as critical targets in the pathological mechanism of depressive disorders. Modulation of InsP3R activity might be useful for the development of new therapeutical strategies for the treatment of mood disorders.

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