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Pierre Meschin, Marie Demion, Olivier Cazorla, Amanda Finan, Jérôme Thireau, et al.. p11 modulates calcium handling through 5-HT₄R pathway in rat ventricular cardiomyocytes. *Cell Calcium*, 2015, 58 (6), pp.549 - 557. 10.1016/j.ceca.2015.08.005 . hal-01761263

HAL Id: hal-01761263

<https://hal.umontpellier.fr/hal-01761263v1>

Submitted on 7 Nov 2018

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p11 modulates calcium handling through 5-HT₄R pathway in rat ventricular cardiomyocytes

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A B S T R A C T

Background: The role of the serotonin receptor 4 (5-HT₄R) pathway in cardiac excitation-contraction coupling (ECC) remains unclear. In the brain, induction of the calcium (Ca²⁺)-binding protein p11 enhances 5-HT₄R translocation and signaling and could therefore be considered as a modulator of the 5-HT₄R pathway in the myocardium. p11 expression is increased by brain-derived neurotrophic factor (BDNF) or antidepressant drugs (imipramine). Thus, we investigated whether p11 regulates the 5-HT₄R pathway in the heart in physiological conditions or under pharmacological induction and the effects on calcium handling.

Methods and results: p11 expression was induced *in vivo* in healthy Wistar rats by imipramine (10 mg/kg/21 days) and *in vitro* in left ventricular cardiomyocytes exposed to BDNF (50 ng/ml/8 h). Cell shortening and real-time Ca²⁺ measurements were processed on field-stimulated intact cardiomyocytes with the selective 5-HT₄R agonist, prucalopride (1 μM). Both imipramine and BDNF-induced cardiomyocyte p11 expression unmasked a strong response to prucalopride characterized by an increase of both cell shortening and Ca²⁺ transient amplitude compared to basal prucalopride associated with a high propensity to trigger diastolic Ca²⁺ events. Healthy rats treated with BDNF (180 ng/day/14 days) exhibited a sustained elevated heart rate following a single injection of prucalopride (0.1 mg/kg) which was not observed prior to treatment.

Conclusions: We have identified a novel role for p11 in 5-HT₄R signaling in healthy rat ventricular cardiomyocytes. Increased p11 expression by BDNF and imipramine unraveled a 5-HT₄R-mediated modulation of cardiac Ca²⁺ handling and ECC associated with deleterious Ca²⁺ flux disturbances. Such mechanism could partly explain some cardiac adverse effects induced by antidepressant treatments.

Keywords:

Serotonin receptor 4
S100A10

Calcium waves

Brain-derived neurotrophic factor

Antidepressant

1. Introduction

Cardiac activity is mainly dependent on calcium (Ca²⁺) cycling and tightly regulated by the autonomic nervous system and chronic disturbances of these two factors may lead to major cardiac dysfunction [1,2]. In addition to catecholamines,

serotonin (5-hydroxytryptamine [5-HT]) is involved in the physiology of the healthy [3] and pathologic human myocardium as described in heart failure [4] and atrial fibrillation where it promotes arrhythmogenicity [5,6]. Although 5-HT is likely to induce several well identified physiological cardiovascular effects in human and pig [4–6], it has been shown to be inactive on ventricular cardiomyocytes in rodents [7,8]. In the last decade, Qvigstad *et al.* proposed a new role for 5-HT in ventricles of rats with heart failure. Indeed, six weeks after myocardial infarction, 5-HT produced a positive inotropic effect through G_s-coupled serotonin receptor 4 (5-HT₄R) activation [9,10]. Similarly to β-adrenergic receptors, 5-HT₄R stimulation increases cytoplasmic cAMP production through adenylate cyclase activity which promotes cAMP-dependent protein kinase A (PKA) activation. PKA phosphorylates key participants of Ca²⁺ handling hence positively enhancing cardiac excitation-contraction coupling (ECC) [1].

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Consistent with this, 5-HT₄R activation has been shown to induce phosphorylation of L-type Ca²⁺ channels (LTCC), troponin I or phospholamban (PLB) in the failing rat heart [11].

In the mouse brain, 5-HT₄R interacts with a small 11 kDa protein, p11 (or S100A10) which increases the translocation of the receptor to the plasma membrane improving 5-HT₄R signaling demonstrated by increased cAMP production [12]. p11 is a member of the S100 protein family which is the largest family of Ca²⁺-binding proteins. Nevertheless, unlike others S100 proteins, amino acid replacements in the p11 Ca²⁺-binding loops make it unable to bind Ca²⁺ but leads to a permanent activated state of p11 [13]. p11 is highly expressed in the lung, intestine and kidney but poorly in the heart [14] where its role has yet to be elucidated. In vessels, p11 is known to play a crucial role in the regulation of plasmin activity as a receptor for plasminogen and tissue-type plasminogen activator (tPA) [15] or in the secretion of endothelial von Willebrand factor [16]. Interestingly, p11 participation has emerged in the intracellular trafficking of several ion channels and G-protein coupled receptors such as transient receptor potential (TRP) channels [17] and sodium channels [18] or serotonin receptors such as 5-HT_{1B} [19] or as mentioned above, 5-HT₄R. p11 expression is modulated by nitric oxide [20], transforming growth factor alpha (TGFα) [21], nerve growth factor (NGF) [22] and brain-derived neurotrophic factor (BDNF) [23] or antidepressants drugs such as imipramine [19].

In the present work, we aimed to explore a potential role for p11 in the modulation of 5-HT₄R signaling at the ventricular level of healthy cardiac tissue. Assessment of p11 was conducted in basal conditions or after pharmacological induction. We have identified that p11 acts as a substantial modulator of ventricular cardiomyocyte Ca²⁺ handling and contractility through the 5-HT₄R pathway. Induction of p11 may be enhanced by factors used in depression treatment and is associated with proarrhythmic Ca²⁺ flux disturbances.

2. Methods

2.1. Animals

All procedures were carried out conforming to the Directive 2010/63/EU of the European Parliament and the Council of 22 September 2010 for the protection of animals used for scientific purposes (agreement number: A34-172-38). Animals were housed in a temperature-regulated room (12-h day:12-h night cycle) with access to food and water *ad libitum*. To induce *in vivo* p11 expression in adult healthy hearts, 200–224 g male Wistar rats from Janvier (Le Genest-Saint-Isle, France) were subjected to either I) Subcutaneous BDNF treatment for 14 days (180 ng/day) (B3795, Sigma-Aldrich, St-Quentin-Fallavier, France) or II) Intraperitoneal injection of either Imipramine (10 mg/kg/day) (I0899, Sigma-Aldrich, St-Quentin-Fallavier, France) or saline for 21 days (rats were sacrificed the day after the last injection). Subcutaneous treatment was achieved by the implantation of osmotic pumps (2ML2, Alzet, Charles River, l'Arbresle, France) under general gaseous anesthesia (2.5% isoflurane (Isovet®, Dechra Veterinary Products, Suresnes, France) in 100% oxygen) on a heating pad. Lidocaine was applied on the wound after surgery.

2.2. Electrocardiograms (ECG) telemetry

ECG transmitters (CA-F40, Data Science International (DSI), St. Paul, Minnesota, USA) were intraabdominally implanted in rats placed on a heating table under general gaseous anesthesia (2.5% isoflurane in 100% oxygen). Telemetric ECGs were recorded one week after surgery and analyzed in conscious free moving rats using a Ponemah physiology platform (DSI) to obtain heart rate

and RR intervals as previously described [24]. To assess the cardiac effects of 5-HT₄R stimulation, healthy adult rats received a single intraperitoneal injection of the 5-HT₄R selective agonist prucalopride (0.1 mg/kg in water) (A11771, AdooQ Biosciences, Irvine, California, USA) before and after subcutaneous treatment with BDNF (180 ng/day/14 days). ECGs recordings were started 60 min prior to prucalopride injections and stopped at the end of the nocturnal phase.

2.3. Cell dissociation

Experiments were performed at the single cell level using left ventricular free wall (LV-FW) cardiomyocytes that were enzymatically isolated as previously described [25]. Briefly, hearts were excised and cannulated through the aorta to a retrograde perfusion Langendorff system to remove the blood from the coronary arterial vasculature. Hearts were then perfused with a free Ca²⁺ physiological tyrode solution (116 mM NaCl, 6 mM KCl, 4 mM NaHCO₃, 1.5 mM KH₂PO₄, 1.7 mM MgCl₂, 21 mM HEPES, 20 mM taurine and 12 mM glucose, pH 7.15) containing a permeant protease inhibitor (E-64d, 10 μM, E8640, Sigma-Aldrich, St-Quentin-Fallavier, France) at constant flow perfusion rate. Hearts were then perfused with type IV collagenase (Worthington, Entraigues, France) solution and left ventricular free walls were collected in a Ca²⁺ free solution, dissected and mechanically dissociated. The Ca²⁺ concentration was gradually increased to 1 mM and cells were maintained in a physiological solution at 37 °C for 30 min before further processing. To induce *in vitro* p11 expression, freshly isolated LV-FW myocytes were incubated with 50 ng/ml BDNF for 8 h at 25 °C.

2.4. Quantitative RT-PCR

Total RNA was isolated from snap-frozen intact tissue or isolated LV-FW cardiomyocytes using a Nucleospin total RNA isolation kit (Macherey-Nagel, Hoerd, France) according to the manufacturer's instructions. Total RNA, oligo-dT and random hexamer primers were used to generate cDNA using the Verso enzyme kit (Fisher Scientific, Illkirch, France). To evaluate p11 and 5-HT₄R expressions, real time RT-PCR was performed using gene-specific primers. Reactions were performed using SYBR green master Mix (Roche Applied Sciences, Meylan, France). The *Rpl32* gene expression was used as the housekeeping gene. Relative gene expression was determined by the 2^{-ΔΔCt} method. Primers sequences were designed with the primer design tool from NCBI (NCBI/primer-BLAST) or as previously described [26,27]:

S100a10 (p11) FW: 5'-CGGGGCCAGGTTTCGACAG-3'
ID: 81718 RV: 5'-CCCGTTCCATGAGCACTCTCAGGT-3'
Htr4 FW: 5'-GATGACCCCTACGCATCG-3'
ID: 25324 RV: 5'-AGCACCATGAGGAGAAACGG-3'
Rpl32 FW: 5'-CACCAGTCGGACCGATATGTGAAAA-3'
ID: 28298 RV: 5'-TGTTGTCGATGCCTCTGGGTTT-3'

2.5. Protein analysis

Proteins were extracted from snap-frozen intact tissue or isolated LV-FW cardiomyocytes with a manual polytron® homogenizer. Protein extracts were lysed in 200 μl lysis buffer (20 mM HEPES, 40 mM KCl, 1 mM DTT, 0.3% CHAPS, 1× protease inhibitor SigmaFAST) for 30 min at 4 °C with mixing. Proteins were then separated by SDS-PAGE, blotted onto nitrocellulose membranes (0.2 μm, GE Healthcare, Brumath, France) for 1 h and then blocked with StartingBlock (TBS) blocking buffer (37543, Fisher Scientific, Illkirch, France) for 2 h at room temperature. Membranes were incubated overnight at 4 °C with primary antibodies: p11 antibody (1/1.000, AF2377, R&D systems, Lille, France) or β1-adrenergic receptor (1/200, sc-568, Santa-Cruz, Heidelberg, Germany). Protein

levels were expressed relative to GAPDH (1/60,000, ab8245, Abcam, Cambridge, UK) or Calsequestrin 2 (CASQ2) (1/2,500, PA1-913, Fisher Scientific, Illkirch, France) expressions. Experiments were repeated a minimum of three times for each condition. All immunoblots were visualized and quantified using the Odyssey® infrared imaging system (LI-COR Biosciences, Lincoln, Nebraska, USA) coupled to infrared-labeled anti-goat, anti-mouse or anti-rabbit IgG secondary antibodies (1/30,000 dilution) (LI-COR Biosciences, Courtaboeuf, France).

2.6. Cellular Ca²⁺ transients and shortening measurements

Real time Ca²⁺ measurements was performed on freshly isolated LV-FW myocytes incubated in a physiological tyrode solution (140 mM NaCl, 4 mM KCl, 1 mM MgCl₂, 5 mM HEPES, 1.8 mM CaCl₂ and 11 mM glucose, pH 7.4). Cardiomyocytes were loaded with the ratiometric Ca²⁺ dye, indo-1AM at room temperature during 20 min (2 μM, Life technologies, St-Aubin, France) and cell shortening/Ca²⁺ transients were recorded using electrical-field stimulation (1 Hz). Sarcomere length (SL) and fluorescence wavelengths emitted at 405 nm (F405) and 480 nm (F480) were simultaneously recorded using the IonOptix® system (Milton, USA) coupled to a Zeiss microscope (40× oil, 0.36 μm/pixel) [24]. The approximate [Ca²⁺]_{cytosolic} was obtained by measuring the ratio F405/F480. In all experiments, 5-HT₄R was stimulated by a 30 min incubation with the specific 5-HT₄R agonist, prucalopride (1 μM), or blocked with a 30 min preincubation with the specific 5-HT₄R blocker GR113808 (10 μM) (G5918, Sigma-Aldrich, St-Quentin-Fallavier, France) prior to prucalopride stimulation. Ca²⁺ waves defined by any spontaneous rise in the fluorescence ratio during the diastolic phase (30 s) were considered as proarrhythmogenic cellular activity. In all experiments, at least 10 cells were recorded per animal. Data were analyzed using IonWizard 6.4 software.

2.7. Statistical analysis

N represents the number of animals and *n* the number of cells. Data are presented as mean ± SEM (or mean ± SD for *in vivo* BDNF experiments). Statistical analyses were performed using SigmaStats 3.5 software. Student *t*-test was used to compare two conditions. For BDNF and imipramine experiments, two-way ANOVA was used to, respectively, compare multiple conditions. For paired values comparison, Wilcoxon signed-rank test was used. Fisher's exact test was used for percentages comparison. *P*-value lower or equal to 0.05 indicated a statistically significant difference.

3. Results

3.1. Low expression of p11 is associated with an inactive 5-HT₄R pathway in healthy LV-FW cardiomyocytes.

The expression of p11 was investigated in different regions of the normal heart at the tissue and cellular levels and was compared to tissues known to express high levels of p11 expression such as the small intestine or the brain (Fig. 1). At the transcript level, p11 mRNA was detected by qualitative conventional RT-PCR in atrial and LV-FW tissue as well as in LV-FW single cardiomyocytes (Fig. 1A, upper panel). In the same tissues, 5-HT₄R mRNA was also expressed but in a more modest pattern compared to the small intestine or the brain (Fig. 1A, lower panel). At the protein level, p11 was expressed in both total atrial and ventricular tissues and specifically in LV-FW cardiomyocytes (Fig. 1B, upper panel). It is worth noting that levels of p11 expression in the LV remained very low compared to tissues where p11 is known to be highly expressed or active (Fig. 1B, lower panel). p11 expression was higher in the atria than in the left ventricle where the 5-HT₄R pathway is

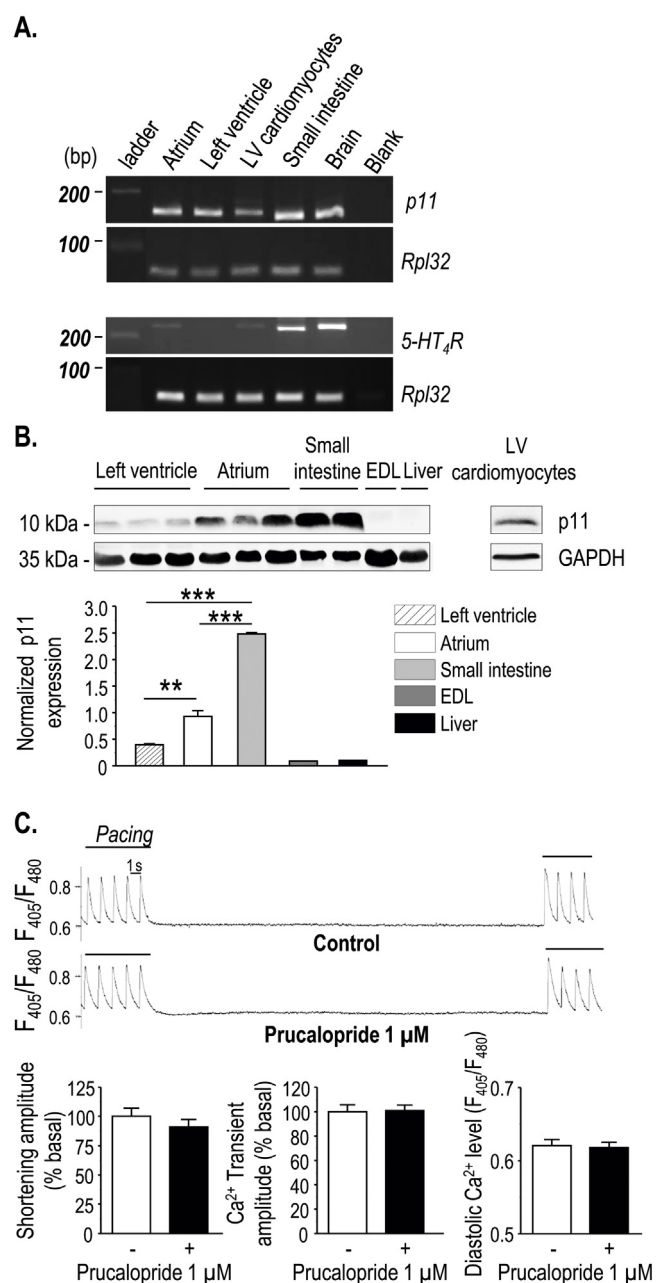
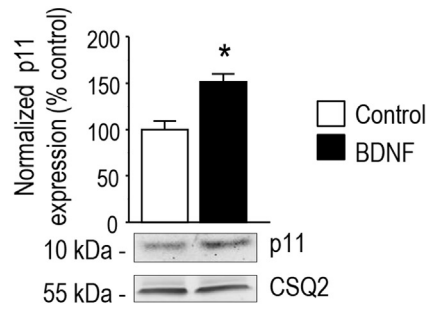
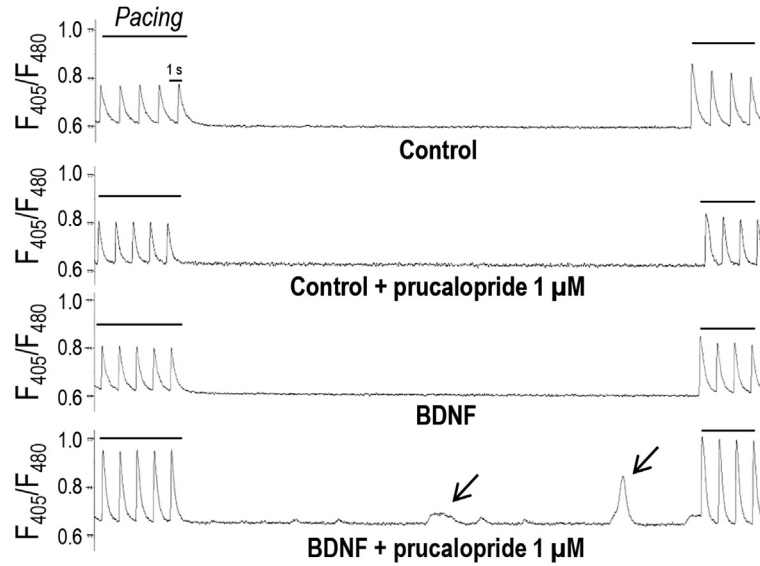
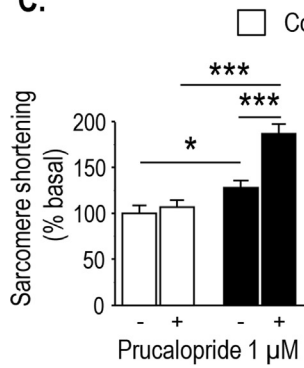
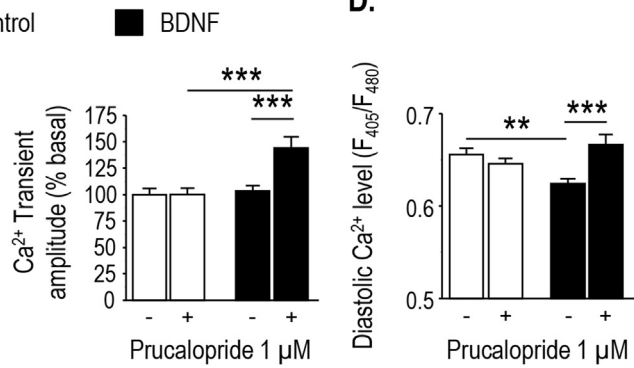
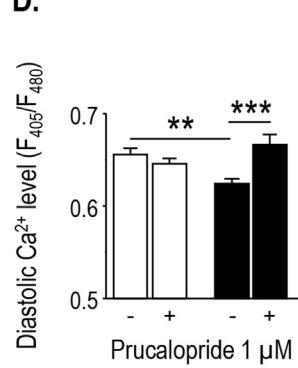
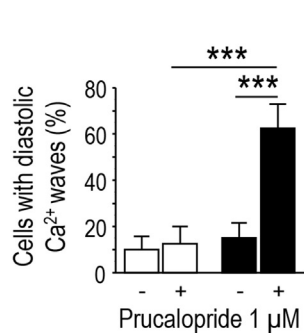
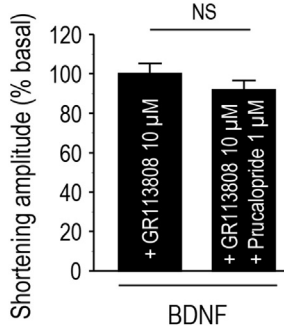
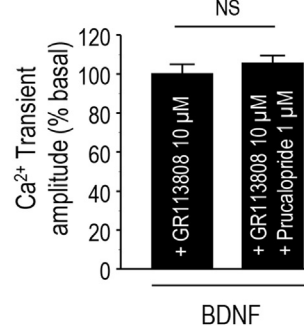


Fig. 1. Physiological p11 expression in cardiac tissue and 5-HT₄R signaling in healthy LV-FW cardiomyocytes. (A) Qualitative mRNA expression in atrium, left ventricle (LV) or adult LV-FW cardiomyocytes from healthy rats assessed by conventional RT-PCR with gene-specific primers for p11 (142 basepair [bp]) (upper panel) or 5-HT₄R (234 bp) (lower panel). The small intestine and the brain served as a positive controls for p11 and 5-HT₄R mRNA expression. Rpl32 gene (64 bp) served as the housekeeping gene (presented samples for p11 or 5-HT₄R mRNA are extracted from the same experiment). (B) p11 protein expression quantified in atrium (*N* = 3), LV (*N* = 3) and LV-FW cardiomyocytes from healthy rats assessed by Western blot. The small intestine (*N* = 2) served as a positive control and both the skeletal muscle (*Extensus Digtorium Longus* [EDL]) (*N* = 1)/the liver (*N* = 1) as negative controls for p11 protein expression. (C, upper panel) Representative traces of indo-1AM ratio obtained in basal condition or with prucalopride (1 μM) in field-stimulated healthy freshly isolated LV-FW cardiomyocytes. (C, lower panels) Effect of prucalopride (1 μM) on SL shortening (lower left panel), Ca²⁺ transient amplitude (lower middle panel) and diastolic Ca²⁺ level (lower right panel) in field-stimulated intact LV-FW cardiomyocytes. *N* stands for the number of animals and *n* for the number of cells: *N* = 3, respectively, *n* = 29 and *n* = 30. White bars represent the basal condition and the black bars, the prucalopride stimulation. Data are presented as mean ± SEM. ***, *p* < 0.01, *p* < 0.001.

A.**B.****C.****D.****D.****E.****F.****F.**

also known to be silent [7] suggesting additional roles of p11 in the former cavity in normal conditions. In this study, we considered several features of ECC (i.e. Ca^{2+} transient and cell shortening) with or without prucalopride stimulation as a functional read out of the activation of the 5-HT₄R pathway. In normal healthy LV-FW cardiomyocytes, activation of the 5-HT₄R pathway by prucalopride (1 μ M) stimulation had no effect on cardiac ECC (Fig. 1C, upper and lower panels). This absence of 5-HT₄R signaling in healthy cells may be due to the concomitant low expression of p11.

3.2. Acute incubation with BDNF induces a 5-HT₄R-mediated positive inotropic response associated with spontaneous diastolic Ca^{2+} waves

Considering the low expression of p11 in physiological conditions, we assessed the potential modulatory role of p11 induction on the 5-HT₄R pathway in the healthy heart. LV-FW cardiomyocytes were incubated with BDNF (50 ng/ml) for 8 h as previously described to induce a substantial increase of p11 protein expression [23] (Fig. 2). Incubation with BDNF at 50 ng/ml significantly increased p11 protein expression by $51.2 \pm 8.7\%$ ($p < 0.05$) compared to untreated cells (Fig. 2A). We then evaluated the effects of elevated p11 expression on cardiac ECC (Fig. 2B–E). p11 induction by BDNF revealed a positive inotropic response to 5-HT₄R stimulation in healthy LV-FW cardiomyocytes. Indeed, we observed that 5-HT₄R stimulation by prucalopride induced a strong inotropic response in BDNF-treated cardiomyocytes characterized by an increase of $79.9 \pm 10.5\%$ (vs. prucalopride alone, $p < 0.001$) and $58.4 \pm 10.5\%$ (vs. BDNF alone, $p < 0.001$) in sarcomere shortening compared to control cardiomyocytes ($6.5 \pm 7.8\%$ prucalopride alone vs. basal, NS) (Fig. 2C, left panel). It is worth noting that BDNF itself increased SL shortening by $28.0 \pm 7.8\%$ ($p < 0.05$) compared to control basal (Fig. 2C, left panel). Similarly, prucalopride increased Ca^{2+} transient amplitude in cells treated with BDNF by $44.0 \pm 10.5\%$ (vs. prucalopride alone, $p < 0.001$) and $41.0 \pm 10.5\%$ (vs. BDNF alone, $p < 0.001$) compared to untreated cardiomyocytes ($3.3 \pm 4.9\%$ prucalopride alone vs. basal, NS) (Fig. 2C, right panel). Diastolic Ca^{2+} level was also increased in BDNF-treated myocytes challenged to prucalopride compared to prucalopride alone or BDNF alone (0.667 ± 0.011 vs., respectively, 0.646 ± 0.006 , $p < 0.001$ and 0.624 ± 0.005 , $p < 0.001$) (Fig. 2D). Interestingly, BDNF significantly decreased diastolic Ca^{2+} level compared to untreated cells in basal conditions (0.624 ± 0.005 vs. 0.656 ± 0.007 , $p < 0.01$). Ca^{2+} reuptake as indexed by Ca^{2+} transient decay was not modified by p11 induction following 5-HT₄R stimulation (Fig. S1A).

Interestingly, 5-HT₄R-mediated enhancement of cardiac ECC was associated with a high propensity to trigger proarrhythmic Ca^{2+} waves during the diastolic phase in BDNF-treated myocytes compared to other conditions ($62.5 \pm 10.3\%$ cells exhibiting diastolic Ca^{2+} waves vs. $12.5 \pm 7.5\%$ prucalopride alone, $p < 0.001$ and vs. $15.0 \pm 6.5\%$ BDNF alone, $p < 0.001$) (Fig. 2E).

The prucalopride-induced positive inotropic response encountered after p11 induction was abolished in presence of the specific 5-HT₄R blocker, GR113808 (10 μ M), confirming the 5-HT₄R pathway involvement in our model (Fig. 2F).

3.3. Chronic imipramine treatment induces cardiomyocyte p11 expression

As several antidepressants have been shown to increase BDNF levels and p11 mRNA expression [19,28], we sought to determine whether chronic treatment with the tricyclic antidepressant (TCA) imipramine would modulate cardiac expression of p11 (Fig. 3). Three weeks treatment with imipramine induced a significant increase by $26.0 \pm 5.0\%$ of p11 mRNA expression in LV-FW cardiomyocytes compared to animals treated with saline (1.26 ± 0.05 vs. 1.00 ± 0.02 , fold change, $p < 0.01$) (Fig. 3A). Although it did not reach significance ($p = 0.11$), levels of p11 protein were increased by $13.6 \pm 5.6\%$ in LV-FW cardiomyocytes after imipramine treatment compared to untreated animals (Fig. 3B). On the other hand, as imipramine is a non-selective monoamine reuptake inhibitor we assessed the effects of chronic imipramine treatment on β_1 -adrenergic receptor expression. We observed that chronic treatment with imipramine did not affect β_1 -adrenergic receptor expression (Fig. 3C).

3.4. Imipramine treatment unravels a 5-HT₄R-mediated positive inotropic response associated with proarrhythmogenic diastolic Ca^{2+} waves

We then assessed the effects of p11 induction by imipramine treatment on cardiac ECC. Induction of p11 by chronic treatment with imipramine was associated with 5-HT₄R-mediated enhancement of cardiac ECC similarly to that observed with the use of BDNF (Fig. 4). A remarkable positive inotropic effect was observed in LV-FW myocytes freshly isolated from imipramine-treated animals following prucalopride challenge (Fig. 4A). Indeed, in these cells, prucalopride stimulation induced an increase of $46.1 \pm 5.9\%$ (vs. prucalopride alone, $p < 0.001$) and $61.6 \pm 5.9\%$ (vs. basal in imipramine-treated animals, $p < 0.001$) in sarcomere shortening compared to LV-FW cardiomyocytes from vehicle-treated animals ($6.5 \pm 7.8\%$ prucalopride alone vs. basal, NS) (Fig. 4B, left panel).

Similarly, in cardiomyocytes isolated from imipramine-treated animals, prucalopride increased Ca^{2+} transient amplitude by $19.4 \pm 4.9\%$ (vs. prucalopride alone, $p < 0.01$) and $38.0 \pm 4.9\%$ (vs. basal in imipramine-treated animals, $p < 0.001$) compared to cardiomyocytes from vehicle-treated animals ($13.1 \pm 3.7\%$ prucalopride alone vs. basal, NS) (Fig. 4B, right panel). In addition, in cardiomyocytes isolated from imipramine-treated animals, diastolic Ca^{2+} level increased compared to prucalopride or imipramine alone (respectively 0.697 ± 0.008 vs. 0.677 ± 0.006 , $p = 0.057$ or 0.674 ± 0.009 , $p < 0.05$) (Fig. 4C). Similarly to BDNF treatment, no effect on Ca^{2+} transient decay was observed after imipramine treatment (Fig. S1B).

Interestingly, as previously observed with BDNF, chronic treatment with imipramine induced a high propensity of LV-FW cardiomyocytes to trigger diastolic Ca^{2+} events compared to other conditions ($52.0 \pm 17.0\%$ cells exhibiting diastolic Ca^{2+} waves vs. $20.5 \pm 2.3\%$ prucalopride alone, $p < 0.01$ or vs. $2.1 \pm 2.1\%$ basal in imipramine-treated animals, $p < 0.001$) (Fig. 4D). In a striking way, this propensity observed after imipramine treatment was similar

Fig. 2. Effects of BDNF on 5-HT₄R-mediated cardiac ECC in healthy left ventricular cardiomyocytes. (A) Normalized p11 protein expression in healthy LV-FW cardiomyocytes after BDNF incubation (50 ng/ml) for 8 h. Untreated cells were also processed 8 h after cell dissociation (control: $N = 3$; BDNF: $N = 3$). (B) Representative traces of indo-1AM ratio obtained with or without prucalopride (1 μ M) stimulation in healthy LV-FW cardiomyocytes in normal conditions or after BDNF incubation (50 ng/ml/8 h). Control cells were also processed 8 h after cell dissociation. The arrows show spontaneous rises of intracellular Ca^{2+} during diastole. (C and D) Effect of prucalopride (1 μ M) on SL shortening (C, left panel), Ca^{2+} transient amplitude (C, right panel) and diastolic Ca^{2+} level (D) in intact field-stimulated LV-FW cardiomyocytes after BDNF incubation (50 ng/ml) for 8 h. (E) Percentage of LV-FW cardiomyocytes exerting diastolic ectopic Ca^{2+} events after BDNF incubation (50 ng/ml) for 8 h. White and black bars represent the cells 8 h after cell dissociation respectively without or with 8 h BDNF incubation. (A–E) Control: $N = 4$, basal, $n = 40$ and prucalopride, $n = 40$; BDNF: $N = 4$, basal, $n = 40$ and prucalopride, $n = 42$. (F) BDNF: $N = 3$, GR113808, $n = 30$ and GR113808 + prucalopride, $n = 32$. N stands for the number of animals and n for the number of cells. Data are presented as mean \pm SEM. ***,**** $p < 0.05$, $p < 0.01$, $p < 0.001$.

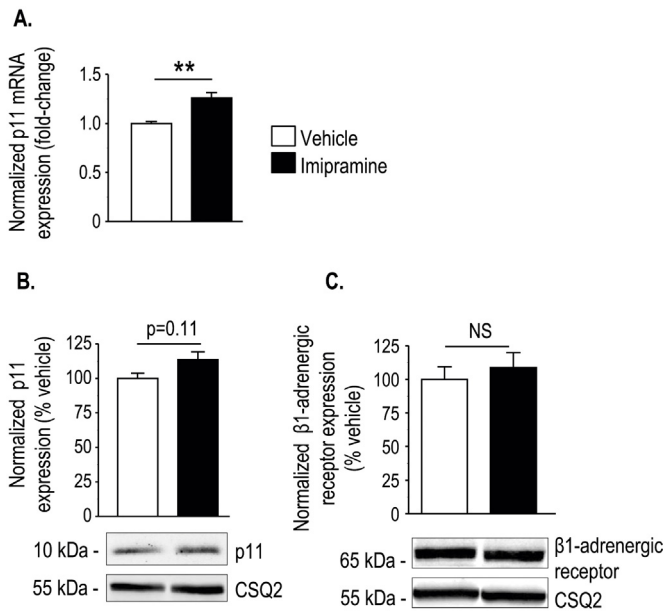


Fig. 3. Effects of imipramine on p11 expression in healthy left ventricular cardiomyocytes. (A–C) Normalized p11 mRNA expression (A) (control: $N=3$; imipramine: $N=3$) and p11 (B) or β 1-adrenergic receptor proteins (C) expressions (control: $N=4$; imipramine: $N=4$) in healthy LV-FW cardiomyocytes from rats treated with saline or imipramine (10 mg/kg/day/ip) for 21 days. ** $p < 0.01$.

to another proarrhythmogenic context obtained after β -adrenergic receptors stimulation with non-selective β -agonist, isoprenaline (10 nM) ($76.0 \pm 9.3\%$ cells exhibiting diastolic Ca^{2+} waves) (Fig. 4D).

3.5. Chronic BDNF treatment induces a sustained chronotropic response to 5-HT₄R stimulation in healthy animals

Regarding the deleterious effects on Ca^{2+} homeostasis caused by p11 induction and 5-HT₄R stimulation *in vitro*, we evaluated whether p11 induction would modulate heart function through 5-HT₄R pathway enhancement *in vivo*. ECG were recorded in free-moving conscious animals allowing heart rate measurement (obtained by RR intervals assessment) in healthy adult rats treated with BDNF for 14 days and undergoing prucalopride injection before and after the treatment (day 0 and day 14) (Fig. 5). Before and after BDNF treatment, an increased heart rate was observed during 25 min after prucalopride injection. This effect may be due to injection-dependent stress or to potential non-cardiac effects of prucalopride resulting in an increase of heart rate (Fig. 5A).

On the other hand, 2 weeks of BDNF treatment allowed a sustained chronotropic response to prucalopride injection for 60 min in the same rats compared to before treatment (417 ± 2 beats/min vs. 393 ± 5 beats/min, $p < 0.001$) (Fig. 5B, left panel). Indeed, whereas heart rate returned to a physiological range before BDNF treatment by 25 min following prucalopride injection, a significant sustained increase in heart rate was observed beyond 25 min in rats after they were treated with BDNF (406 ± 2 beats/min vs. 366 ± 4 beats/min, $p < 0.001$) (Fig. 5B, right panel).

4. Discussion

The physiopathological cardiac effects of the 5-HT₄R pathway remain poorly understood. The 5-HT₄R partner p11 has a clear role in 5-HT₄R signaling in the mouse brain [12,19,23] but its role in the cardiac tissue had not been investigated yet. Here, we have demonstrated that p11 may act as a critical modulator of Ca^{2+} handling and ECC in rat ventricular cardiomyocytes through the activation

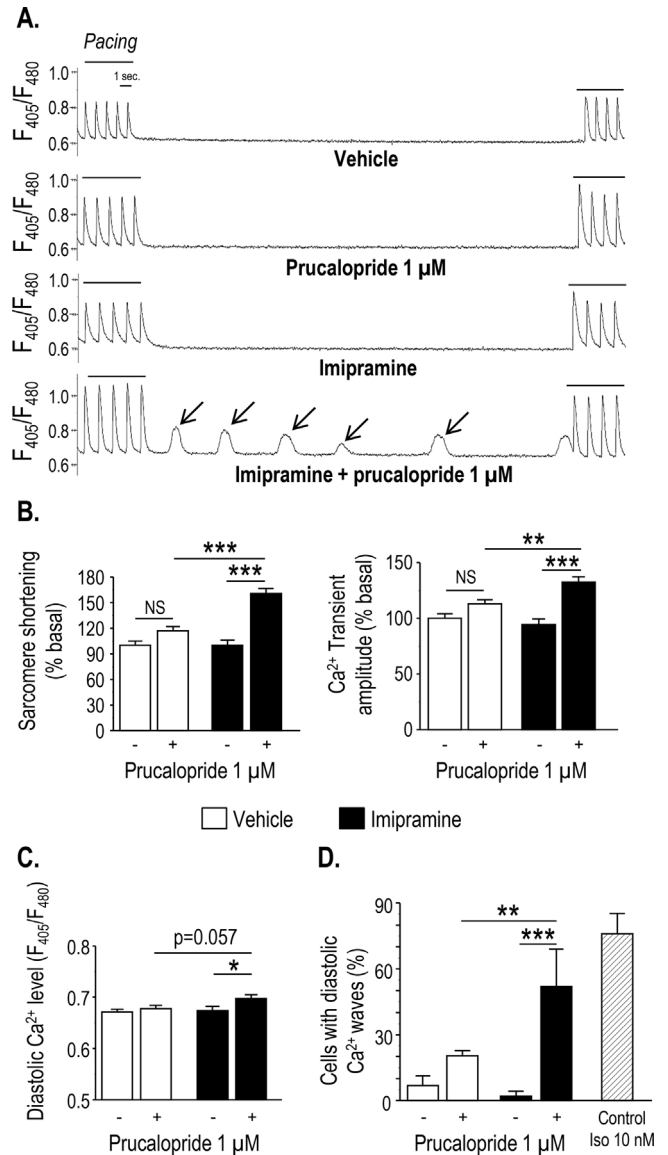


Fig. 4. Effects of imipramine on 5-HT₄R-mediated cardiac ECC in healthy left ventricular cardiomyocytes. (A) Representative traces of indo-1AM ratio obtained with or without prucalopride (1 μM) stimulation in healthy LV-FW cardiomyocytes freshly isolated from rats treated with saline or imipramine (10 mg/kg/21 days/ip). The arrows show spontaneous rises of intracellular Ca^{2+} during diastole. (B and C) Effect of prucalopride (1 μM) on SL shortening (B, left panel), Ca^{2+} transient amplitude (B, right panel) and diastolic Ca^{2+} level (C) in intact field-stimulated LV-FW cardiomyocytes from rats treated with imipramine (10 mg/kg/day/ip) or saline for 21 days. (D) Percentage of LV-FW cardiomyocytes exerting diastolic Ca^{2+} ectopic events isolated from rats treated with imipramine (10 mg/kg/day/ip) or saline for 21 days or isolated from healthy rats and challenged with 10 nM isoprenaline ($n=50$ cells). Data are presented as mean \pm SEM. N stands for the number of animals and n for the number of cells: control: $N=4$, basal, $n=43$ and prucalopride, $n=44$; imipramine: $N=4$, basal, $n=44$ and prucalopride, $n=43$. *** $p < 0.05$, $p < 0.01$, $p < 0.001$.

of the cardiac 5-HT₄R pathway. Although low levels of p11 expression may be insufficient to induce 5-HT₄R activity in physiological conditions, we have unraveled conditions in which this pathway is enhanced.

In our study, we considered several parameters of Ca^{2+} handling obtained with prucalopride stimulation as a read-out of the 5-HT₄R pathway activation. Based on the literature, assessment of the cellular expression or localization of the cardiac 5-HT₄R is strongly limited due to technical issues related to the low density of the receptors. We have therefore concluded that cardiac

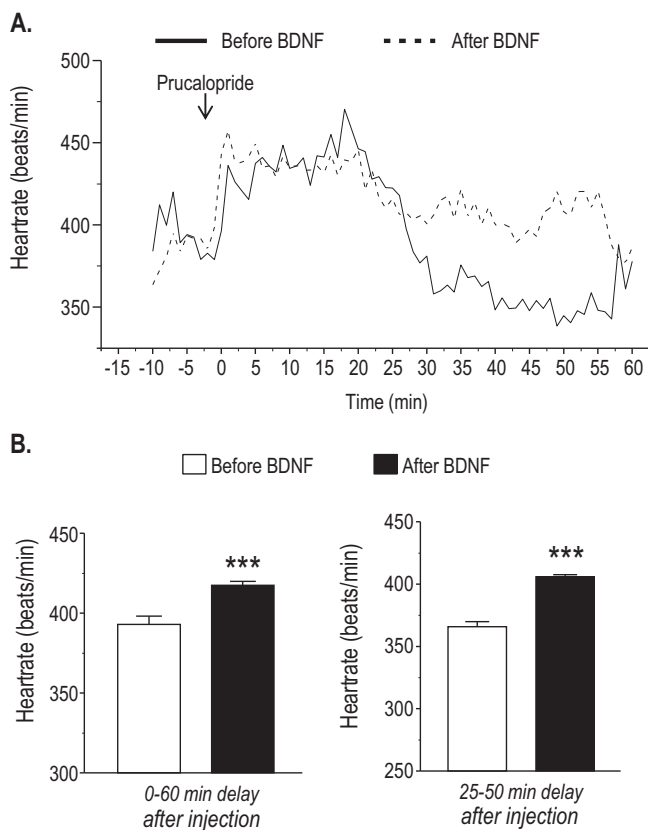


Fig. 5. Effects of BDNF treatment on 5-HT₄R-mediated cardiac function in healthy hearts. (A) Effect of prucalopride injection (0.1 mg/kg/ip) before and after BDNF treatment (180 ng/day/14 days/sc) on heart rate. (B) Averaged heart rates during 60 min (left panel) or 25–50 min (right panel) following prucalopride injection before and after BDNF treatment. The arrow represents the time of injection. Straight and dashed lines, respectively, represent heart rates before and after BDNF treatment. Data are presented as mean \pm SEM. $N = 6$ animals. *** $p < 0.001$.

5-HT₄R pathway-mediated effects on Ca²⁺ handling are a feature of cardiomyocyte expression of p11, responses to prucalopride stimulation and the role of p11 in the 5-HT₄R translocation previously described in the literature [12]. Taking advantage of the rodent model in which the 5-HT₄R pathway is functionally silent in the ventricle in normal conditions, we now demonstrate that p11 induction in healthy cardiomyocytes increases the activity of the cardiac 5-HT₄R pathway and thus modulates Ca²⁺ flux in these cells.

BDNF induces p11 expression but also exerts direct effects on the heart, especially in coronary heart disease [29,30]. BDNF acts mainly *via* the tropomyosin-related kinase B (trkB) receptor [31]. Indeed, in a recent study, Feng *et al.* showed that BDNF was active on rodent cardiomyocytes through the trkB pathway activation [32]. According to these evidences, we hypothesized that increasing BDNF levels may modulate cardiac p11-dependent 5-HT₄R signaling. Although, in rodents, the 5-HT₄R pathway remains to be silent at the ventricular level in non-pathological states in contrast with findings in failing animals [9], BDNF-dependent p11 induction exerted remarkable functional effects on cardiac ECC following 5-HT₄R stimulation.

The elevated p11 expression induced 5-HT₄R activity as a possible threshold enabling a global effect on Ca²⁺ handling at the cellular level even in physiological conditions. This enhancement observed at the cellular level was encountered *in vivo* as the 5-HT₄R pathway stimulation impacted heart rate after BDNF treatment. As 5-HT₄R is the only serotonin receptor subtype expressed in the human sinoatrial node, whose stimulation leads to long-lasting

tachycardia [33], BDNF may also enhance 5-HT₄R activity in this compartment resulting in the increased heart rate observed in our study. We have shown that 5-HT₄R-mediated enhancement of cardiac ECC was associated with a high propensity to trigger proarrhythmic diastolic Ca²⁺ events at the cellular level which are likely to be a cause of delayed afterdepolarizations (DADs) that may further trigger ventricular arrhythmias and lead to sudden cardiac death [34].

The commonly used TCA imipramine promoted similar effects on p11 expression and 5-HT₄R-related Ca²⁺ handling. Imipramine was previously shown to increase p11 mRNA expression in the mouse forebrain and cortex [19]. Interestingly, it exerts a similar pattern of p11 mRNA induction in the heart and in the brain (30% increase). However, based on the literature, p11 induction by imipramine at the protein level has yet to be investigated. Herein, we have shown that imipramine may increase cardiac p11 expression leading to an enhancement of 5-HT₄R-mediated Ca²⁺ handling in ventricular cardiomyocytes. Considering the features of cardiac ECC after 5-HT₄R stimulation in animals treated with imipramine, we assumed that I_{CaL} activity is largely responsible for the modulation of Ca²⁺ handling as the 5-HT₄R stimulation is known to strongly enhance LTCC activity [11]. Enhanced LTCC activity may induce intracellular Ca²⁺ overload and cause elevated diastolic [Ca²⁺]_i. Moreover, supported by a body of evidence transposable to the cardiac 5-HT₄R pathway [35–37], a potential role of leaky RyR2 in the occurrence of diastolic aberrant Ca²⁺ release such as Ca²⁺ waves is expected to be preponderant in our model.

Although BDNF induced a stronger increase of p11 protein than imipramine, the effects of the 5-HT₄R pathway in both conditions seemed relatively similar as observed by cardiac Ca²⁺ handling and ECC, suggesting a non-related-p11 mechanism involved with imipramine treatment. As imipramine is a non-selective monoamine reuptake inhibitor, the inhibition of norepinephrine reuptake may potentiate 5-HT₄R activity possibly by increasing the desensitization of β -adrenergic receptors due to chronic stimulation without affecting their expression as demonstrated here. Hence, chronic stimulation of both PKA-related pathways (5-HT₄R and β -adrenergic pathways) may induce remodeling of Ca²⁺ handling key actors in the heart.

Although the literature is quite controversial whether imipramine increases [38–40] or not [41] BDNF levels, it has been shown to activate trkB signaling independently from BDNF by physically interacting with the receptor [42]. This supports a potential similar mechanism in our study. While the 5-HT₄R pathway is functionally active in the normal human heart at both atrial and ventricular levels, the present data may be relevant assuming that the 5-HT₄R response would be enhanced by p11 induction due to antidepressant treatments [43,44].

Depression and cardiovascular disease have been reciprocally linked but the physiopathological features are not clearly defined. These diseases have been predicted to be the two most severe mortality factors in the coming years [45]. Selective serotonin reuptake inhibitors (SSRI), noradrenaline reuptake inhibitors or TCA are the mainstream medications for the treatment of depression [46]. Contrary to their significant beneficial effects on depression symptoms, antidepressant treatments may also induce cardiac toxicity or rhythmic disorders such as prolonged QT interval [47,48], a potential substrate for arrhythmias. BDNF levels have been shown to be restored following chronic treatment with several classes of antidepressants [38,39,49] as well as p11 levels [19]. Here, we suggest that during antidepressant treatment, p11 induction by TCA or BDNF increases cardiac 5-HT₄R pathway activity, potentially overstimulated due to 5-HT-reuptake inhibition carried out by antidepressant drugs. Thus, sustained 5-HT₄R stimulation in cardiomyocytes enhancing Ca²⁺ handling and generating diastolic Ca²⁺ waves could provide conditions for cardiac remodeling

overtime and occurrence of arrhythmias which may explain and/or contribute to the antidepressant-related cardiotoxicity.

5. Conclusion

This study is the first to show a role for p11 in the 5-HT₄R-mediated modulation of cardiac Ca²⁺ handling and ECC in the myocardium. Induction of p11 protein by several factors involved in depression management such as the neurotrophin BDNF or the TCA imipramine unravels strong 5-HT₄R-mediated effects on Ca²⁺ handling associated with diastolic Ca²⁺ abnormalities known to be deleterious. Although the physiological activity of the cardiac 5-HT₄R pathway between human and rodents still remains a point of discrepancy, these results obtained in a rodent model may interestingly translate to the effects of a potential p11-dependent enhanced 5-HT₄R pathway on Ca²⁺ handling in humans treated with TCA. Considering the emerging links between antidepressants, BDNF, p11 and 5-HT₄R, our data suggest that the modulation of Ca²⁺ handling in ventricular cardiomyocytes through enhanced 5-HT₄R pathway and p11 induction could partly explain the antidepressant-mediated cardiotoxicity.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

This work was supported by funding from the French Institut National de la Santé Et de la Recherche Médicale (INSERM), 'Fondation de France (2013 00038586)', and the French Fondation pour la Recherche Médicale (FRM).

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