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Cyclin-dependent kinase 5 modulates the P2X2a receptor channel gating through phosphorylation of C-terminal threonine 372

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Abstract

The purinergic P2X2 receptor (P2X2R) is an ATP-gated ion channel widely expressed in the nervous system. Here, we identified a putative Cdk5 phosphorylation site in the full-size variant P2X2aR (^{372}TPKH^{375}), which is absent in the splice variant P2X2bR. We therefore investigated the effects of Cdk5 and its neuronal activator, p35, on P2X2aR function. We found an interaction between P2X2aR and Cdk5/p35 by co-immunofluorescence and co-immunoprecipitation in HEK293 cells. We also found that threonine-phosphorylation was significantly increased in HEK293 cells co-expressing P2X2aR and p35 as compared to cells expressing only P2X2aR. Moreover, P2X2aR-derived peptides encompassing the Cdk5 consensus motif were phosphorylated by Cdk5/p35. Whole-cell patch-clamp recordings indicated a delay in development of use-dependent desensitization (UDD) of P2X2aR but not of P2X2bR in HEK293 cells co-expressing P2X2aR and p35. In *Xenopus* oocytes, P2X2aRs showed a slower UDD than in HEK293 cells and Cdk5-activation prevented this effect. A similar effect was found in P2X2a/3R heteromeric currents in HEK293 cells. The P2X2aR-T372A mutant was resistant to UDD. In endogenous cells, we observed similar distribution between P2X2R and Cdk5/p35 by co-

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Conflict of Interest Statement

The authors had no conflicts of interest to declare.

localization using immunofluorescence in primary culture of nociceptive neurons. Moreover, co-immunoprecipitation experiments showed an interaction between Cdk5 and P2X2R in mouse trigeminal ganglia. Finally, endogenous P2X2aR-mediated currents in PC12 cells, and P2X2/3R mediated increases of intracellular Ca^{2+} in trigeminal neurons were Cdk5-dependent, since inhibition with roscovitine accelerated the desensitization kinetics of these responses. These results indicate that the P2X2aR is a novel target for Cdk5-mediated phosphorylation, which might play important physiological roles including pain signaling.

Introduction

The Cyclin-dependent kinase 5 (Cdk5) is an emergent and essential kinase involved in the physiology of sensory pathways [41; 52]. Cdk5 plays key roles in brain development and function, including neuronal migration, membrane transport, axon guidance, and pain signaling [19; 20; 52]. Although Cdk5 is ubiquitously expressed, it is mostly active in post-mitotic neurons, where its specific activators p35 and p39 are mainly expressed [19; 30; 55]. Cdk5 is a proline-directed serine/threonine kinase that phosphorylates many proteins including cytoskeletal proteins, ion channels and regulatory proteins [2]. We reported earlier that Cdk5 regulates pain signaling in nociceptive neurons of the dorsal root ganglion (DRG) and trigeminal ganglion (TG) [41; 46; 47; 52]. Cdk5 activity increases during peripheral inflammation, by the action of cytokines such as TNF-α [47; 53; 57] and TGF-β1 [54; 56]. Moreover, we found that Cdk5 phosphorylates the transient receptor potential vanilloid 1 (TRPV1) in Thr407, a key ion channel implicated in pain, increasing its function [25; 33; 40; 47; 54; 56]. Remarkably, TRPV1 is phosphorylated in Ser116 and Thr370 by PKA [35], and its dephosphorylation is important for the desensitization process [36]. Interestingly, other channels such as the purinergic P2X receptor (P2XRs) also contribute to pain signaling. However, their functional modulation by Cdk5 has not been deeply analyzed.

P2XRs are a family of extracellular ATP-gated ionic channels, widely expressed in different tissues and involved in several physiological and pathophysiological processes [7; 49]. The P2X2R is one of the most abundant subtypes in the nervous system and its subunits can form homomeric or heteromeric channels with P2X3R subunit (P2X2/3R), which are expressed in nociceptive neurons and their activation is involved in pain signaling [8; 9]. Several functional splice variants of P2X2R exists [13], that differ in their desensitization properties; e.g. the shorter variant P2X2bR, lacks a 69 amino acids in the intracellular C-terminal domain and desensitizes faster than the full length P2X2aR [5; 28; 48]. Compared to other P2XR subtypes, P2X2Rs exhibit slow desensitization profile, which depends on agonist and Ca^{2+} concentration, and in N- and C-terminal residues/domains [13; 14; 27]. The gating properties of P2X2R are also affected by protons, divalent metals [11; 34], reactive oxygen species [12], steroid hormones [18], and membrane phosphoinositides [22], all these molecules act as allosteric regulators. Additionally, phosphorylation of P2X2R by PKA and PKC also regulates its gating properties [3; 16].

Here, we show that Cdk5 regulates the activity of P2X2R, by phosphorylation of Thr372, a residue exclusively present in the full-length P2X2aR, but absent in P2X2bR. Using confocal microscopy, biochemical and electrophysiological techniques, we found that

Cdk5/p35 physically interacts with P2X2R in heterologous overexpression systems, primary cultures of TG and DRG neurons, and in PC12 cells that endogenously express Cdk5/p35 and P2X2R. We also found that Cdk5/p35 can phosphorylate P2X2aR, regulating the gating properties that results in desensitization changes, in both heterologous and endogenous systems. These findings suggest that the P2X2aR is a new target for Cdk5-mediated phosphorylation, which might play an important role in pain signaling and therefore could be a potential novel target for developing pain therapies.

Methods

P2X2R and P2X2/3R expression in HEK293 cells and *Xenopus laevis* oocytes

HEK293 cells (ATCC# CRL-1573) were grown in Dulbecco's Modified Eagle Medium (DMEM) containing 10% of fetal bovine serum (FBS) and pen/strep (Invitrogen, Carlsbad CA). For Western blot and co-immunoprecipitation analysis, HEK293 cells were transiently co-transfected during 24 h with rat P2X2aR-pIRES-GFP or P2X2bR-pIRES-GFP in combination with mouse p35 and mouse Cdk5 vectors by using Lipofectamine 2000 reagent (Invitrogen, Carlsbad CA). For immunofluorescence experiments, HEK293 cells were grown on 12-mm coverslips treated with poly-L-lysine and then transfected for 24 h with rat P2X2aR-GFP fusion protein, mouse p35 and Cdk5 plasmids. For patch-clamp experiments, HEK293 cells were grown on 35-mm dishes at a density of 500,000 cells per plate and transfected with rat P2X2aR-pIRES-GFP, P2X2bR-pIRES-GFP, or P2X2aR-pIRES-GFP T372A mutant receptor with or without p35 vectors. For the experiments with the heteromeric P2X2/3R we co-transfected rat P2X2aR-pIRES-GFP, rat P2X3R-pIRES-GFP and p35 vector and for the isolation of the heteromeric currents, we used the specific agonist α,β-methylene ATP (α,β-meATP). For experiments in *Xenopus laevis* oocytes, a segment of the ovary was surgically removed from female frogs under anesthesia with benzocaine 0.05 % and oocytes were manually defolliculated and incubated with collagenase II (1 mg/ml). Each oocyte was injected intranuclearly with 3–5 ng of cDNA coding for the different P2X2Rs alone or combined with the p35 plasmid. Patch-clamp experiments for HEK293 cells and two-electrode voltage-clamp experiments for *Xenopus* oocytes were performed 18–48 h after transfection/injection.

Primary culture of neurons from mouse TG and DRG and rat TG

Nociceptive neurons were cultured as described previously [47]. Briefly, TG and DRG were dissected out from mice or rat of 2–4 month old and incubated with collagenase XI (0.66 mg/mL) and dispase II (3 mg/mL) (Sigma-Aldrich) in an INC-mix solution (NaCl 155 mM; K_2HPO_4 1.5 mM; HEPES 10 mM; glucose 5 mM; at pH 7,4). Enzymatic digestion was performed for 45 min at 37°C in 5% CO_2, and cells were cultured in minimum essential media (MEM) supplemented with 10% FBS, Pen/Strep 100 mg/mL, MEM-vit (Invitrogen, Carlsbad CA). Cells were plated on 12-mm poly-L-lysine-coated glass coverslips and cultured for 2 days. Animal experiments were conducted in accordance with the principles and procedures of the National Institutes of Health Guidelines for the Care and Use of Laboratory and the Bioethical Committee of Universidad Católica del Norte, Coquimbo, and the Ethics Committee of the Biology Department, Faculty of Sciences, Universidad of Chile, Santiago, Chile.

PC12 cell culture

The PC12 cell line (ATCC# CRL-1721) was grown in DMEM containing 10% FBS, 5% horse serum (HS) and pen/strep. For Immunofluorescence and electrophysiological recordings experiments, PC12 cells were plated on 12-mm coverslips treated with poly-L-lysine. For Cdk5 inhibition experiment, PC12 cells were treated with roscovitine 30 μM during 6 and 24 h before electrophysiological recordings. To evaluate the current of P2X2aR or P2X2aR T372A mutant, we transfected those vectors in PC12 cells and current was measured 24 h later.

Western blot analysis

Protein extracts from HEK293 cells, PC12 cells or from mouse TG and DRG were obtained in T-PER buffer (Pierce, Rockford, IL) with Complete Mini protease inhibitor cocktail tablets and PhosSTOP phosphatase inhibitor cocktail tablets (Roche Diagnostic, Indianapolis, IN). Protein concentration was determined using the Bradford Protein Assay (Bio-Rad, Hercules, CA). Proteins were separated in SDS-PAGE gels and transferred to nitrocellulose membranes (Invitrogen, Carlsbad, CA). Membranes were soaked in blocking buffer (5% nonfat dry milk in Tris-Buffered Saline (TBS) with 0.05% Tween-20 (TBS-T)) for 1 h at room temperature, and then incubated overnight at 4°C, with primary antibody diluted in 1% nonfat dry milk blocking buffer. The membranes were washed in TBS-T and incubated for 1 h at room temperature with the secondary antibodies diluted in 1% nonfat dry milk blocking buffer. Immunoreactivity was detected by using Super-Signal West Pico or Dura Chemiluminescent Substrate (Thermo Scientific, Rockford, IL). Western blots were performed using P2X2R rabbit antibody #APR-003 and P2X3R rabbit antibody #APR-016 from Alomone Labs (Jerusalem, Israel); Cdk5 rabbit antibody C8, Cdk5 mouse antibody DC17, p35 rabbit antibody C19, and p35 goat antibody A18 from Santa Cruz Biotechnology (Dallas, TX); Phospho-(Thr) MAPK/CDK substrate mouse antibody #2321, GFP rabbit antibody #2555, and p35 #2680 were obtained from Cell Signaling Technology; and α-tubulin antibody from Sigma-Aldrich (San Louis, MO) was used as loading control. We also used a control peptide antigen from P2X2R sequence (Alomone Labs, Jerusalem, Israel) as negative control for P2X2R Western blot and immunofluorescence experiments. The optical densities of the bands were quantified using an image analysis system with ImageJ 1.46r software (NIH, Bethesda, MD).

Co-immunoprecipitate assay

To evaluate the putative interaction between P2X2aR and Cdk5/p35 we immunoprecipitated protein extracts (1 mg) from HEK293 cells co-transfected with P2X2aR-IRES-GFP and p35; or P2X2aR-IERS-GFP and Cdk5 plasmids, by using with 2 μg of rabbit p35 antibody (C19) or mouse Cdk5 antibody (DC17), respectively. In addition, we immunoprecipitated 350 μg of protein extracts from mouse TG by using 2 μg of rabbit Cdk5 (C8) antibody. The incubation of antibodies was performed for 4 h at 4°C with stirring followed the addition of 25 μl of Protein A/G PLUS-agarose beads (Santa Cruz Biotechnology, Dallas, TX) incubated overnight at 4°C with stirring. Immunoprecipitated proteins (IP) were washed by centrifugation at 2,500 rpm at 4 °C for 3 times in cold PBS. Loading buffer was added to IPs and they were electrophoresed on SDS-PAGE (protocol continues as Western blot analysis).

To evaluate threonine phosphorylation of P2X2aR mediated by Cdk5/p35, we immunoprecipitate 300 μg of protein extracts from HEK293 cells co-transfected with P2X2aR-IRES-GFP with or without p35 plasmid by using 2 μg of P2X2R rabbit antibody #APR-003 from Alomone Labs and followed a Western blot against Phospho-(Thr) MAPK/CDK substrate mouse antibody #2321. We used normal IgG goat antibody (#AB-108-C, R&D System) as IgG control. As negative control we used protein extracts from HEK293 cells overexpressing P2X2aR and p35 or Cdk5 incubated only with Protein A/G PLUS-agarose beads without primary antibody.

Immunofluorescence analysis

We analyzed the co-localization of P2X2aR with Cdk5/p35 and the cellular localization of P2X2aR-T372A vector in HEK293 cells co-transfected with those vectors. Additionally, we analyzed the P2X2R and p35 endogen expression in PC12 cells. Furthermore, we examined the co-localization of endogen P2X2R and P2X3R, with p35 and Cdk5 in primary culture of neurons from mouse TG and DRG. Thus, transfected HEK293 cells, PC12 cells or primary cultured neurons were washed with warm PBS for 5 min and fixed with a 4% PFA-4% sucrose solution in PBS at 37°C for 20 min. After fixation, cells were washed 3 times with PBS and permeabilized for 5 min with a 0.2% Triton X-100 solution in PBS. After washout with PBS, cells were blocked with a 5% BSA solution in PBS at room temperature for 1 h. Corresponding primary antibodies were used at following concentrations: anti-Cdk5 rabbit C8 antibody (1:100) and anti-Cdk5 mouse DC17 (1:100) antibody (Santa Cruz Biotechnology); anti-p35 goat A18 (1:100) and anti-p35 rabbit C19 (1:100) antibody (Santa Cruz Biotechnology); MAP1B goat N-19 (1:100) antibody (Santa Cruz Biotechnology); and anti-βIII tubulin mouse clone G7121 (1:1000) antibody (Promega); P2X2R rabbit #APR-003 (1:250) antibody (Alomone Labs). All primary antibodies were diluted in 1% BSA solution and incubated overnight at 4°C. The coverslips were washed with PBS and then incubated for 1 h at room temperature with corresponding secondary antibodies: anti-rabbit conjugated to Alexa Fluor®546, anti-mouse conjugated to Alexa Fluor®488, and anti-goat conjugated to Alexa Fluor®647 (Molecular Probes, Life Technologies, Grand Island, NY) in combination with Dapi (Thermo Fisher Scientific). Finally, coverslips were washed with PBS, then they were dried and mounted on a slide with FluorSave (Calbiochem) and then were observed using confocal microscopy (LSM 710 Meta Model, Carl Zeiss Microscopy) and processed with the LSM Image Browser (Carl Zeiss Microscopy) software.

Preparation of RNA and reverse transcription polymerase chain reaction (RT-PCR)

Conventional RT-PCR was performed as described previously [47]. Briefly, total RNA was extracted from frozen DRGs and TGs from wild type 5-month-old mice or from PC12 cells by using TRIzol™ Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Total RNA was digested with TURBO™ DNase (Ambion, Austin, TX) for 1 h to remove contaminating genomic DNA, and retrotranscription was carried out with M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA) by using Oligo (dT). We analyzed mRNA levels of p35, Cdk5, P2X2R isoforms, P2X3R and S29 by using conventional RT-PCR. The following primers were used: p35 S: 5′-GCC CTT CCT GGT AGA GAG CTG-3′, p35 AS: 5′-GTG TGA AAT AGT GTG GGT CGG C-3′; Cdk5 S: 5′-GGC TAA AAA CCG GGA AAC TC-3′, Cdk5 AS: 5′-CCA TTG CAG CTG TCG AAA TA-3′; P2X2R common S: 5′-

GGA ACT GTG ACC TGG ACT TG-3′, P2X2R common AS: 5′-CAG ATC CAG GTC TGT AGC TT-3′; P2X2aR S: 5′-CAA GGC ACC CCT CAA GTA GA-3′, P2X2aR AS: 5′-GCT GGT CCT GGG AGT AGT GA-3′; P2X3R S: 5′-CAG GGC ACT TCT GTC TTT GTC-3′, P2X3R AS: 5′-AGC GGT ACT TCT CCT CAT TCT C-3′; S29 S: 5′-GGA GTC ACC CAC GGA AGT TCG G-3′ and S29 AS: 5′-GGA AGC ACT GGC GGC ACA TG-3′.

In vitro phosphorylation of P2X2aR based peptides

We identified a putative consensus motif for Cdk5 phosphorylation in P2X2aR protein sequence by using prediction websites Motif-Scan http://scansite.mit.edu/motifscan_seq.phtml and NetPhos2.0 http://www.cbs.dtu.dk/services/NetPhos/ In order to evaluate potential Cdk5 phosphorylation sites, 10 amino acid residues of P2X2aR peptides were tested in an *in vitro* Cdk5 kinase assay. Recombinant full-length human Cdk5 and p35 (Sigma) were incubated in 50 μl of kinase assay buffer [100 mM Tris-HCl (pH 7.4); 50 mM $MgCl_2$; 5 mM EDTA; 50 μM NaF; 5 μM Na_2VO_3; 5 mM DTT] containing either 10 μg of histone H1 (Sigma) or P2X2aR peptides (mouse P2X2aR, KVRTPRHPSS; human P2X2aR, KVCTPSHPSG; rat P2X2aR, KVRTPKHPSS; and non-related peptide, IGQSPFHGDD). Kinase assays were carried out at 30°C for 60 min by adding 5 μCi of [γ-^{32}P] ATP (0.5 mM). The peptide assay was stopped by adding 10% trichloroacetic acid to precipitate proteins. 20-μl aliquots of trichloroacetic acid supernatant were transferred onto P81 phosphocellulose squares (spotted in duplicates), air-dried, and washed five times for 15 min each in 75 mM phosphoric acid and once in 95% ethanol. After air drying, squares were transferred to vials containing Bio-Safe II scintillation fluid (Research Products International, Mount Prospect, IL) for counting in a Beckman Coulter (Fullerton, CA) scintillation counter (model SL 3801).

Current measurements in HEK293 and PC12 cells

Electrophysiological experiments were performed on cells at room temperature using whole-cell patch-clamp recording techniques. The currents were recorded using an Axopatch 200B patch-clamp amplifier (Molecular Devices) and were filtered at 2 kHz using a low-pass Bessel filter. Patch electrodes, fabricated from borosilicate glass (type 1B150F-3; World Precision Instruments), using a Flaming Brown horizontal puller (P-87; Sutter Instruments), were heat polished to a final tip resistance of 2–4 MΩ. All current records were captured and stored using the pClamp 9 software packages in conjunction with the Digidata 1322A analog-to-digital converter (Molecular Devices). Unless otherwise specified, patch electrodes were filled with a solution containing the following: 142 mM NaCl, 10 mM EGTA, and 10 mM HEPES. The osmolarity of the internal solutions was 305 mOsm. The bath solution (KR-like) contained the following: 142 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose, and 10 mM HEPES. The Ca^{2+}-free solution contained 145 mM NaCl, 3 mM KCl, 10 mM glucose, and 10 mM HEPES. In all cases pH was adjusted to 7.35, and the osmolarity of those solutions was 295–305 mOsm. ATP was daily prepared in bath buffer and applied using a rapid solution changer system (RSC-200; Biologic Science Instruments). The current responses were recorded from single cells clamped at –60 mV. Most of the data was collected from recordings of single cells stimulated with 10 μM of ATP with a washout interval of 4 min between each application and normalized to the highest

current amplitude. For PC12 recordings of endogenous P2X2R-mediated currents, we used similar conditions to that used for HEK293 cells recordings, but the intracellular solution contained 5 mM ATP and 0.05 mM EGTA in order to observe the endogenous phosphorylation of the P2X2R by Cdk5. Roscovitine (30 μM) was incubated for 6 and 24 h before experiments.

Current measurements in Xenopus oocytes

Oocytes were bathed in Barth's solution [88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 10 mM HEPES, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, and 0.91 mM $CaCl_2$, pH 7.5] supplemented with 10 IU/L penicillin/10 mg streptomycin and 2 mM pyruvate, oocytes were clamped at −60 mV using the two-electrode voltage-clamp configuration with an OC-725C clamper (Warner Instruments). ATP-evoked currents were recorded after regular 1–1000 μM ATP applications with washout periods ranging from 3 to 15 min depending on ATP concentration. For the experiments with allosteric modulators, oocytes expressing P2X2aR or P2X2aR-T372A vectors were challenged with 10 μM ATP that resulted in small, non-desensitizing currents. Copper, zinc and PPADS solutions (selective P2XR antagonist) at a concentration of 10 μM were pre-applied for 1 min and then were co-applied with ATP; the currents induced by this application were compared to that induced by ATP alone. For the pH experiments, the ATP containing solution was adjusted to a pH 6.5 and the currents induced by this solution were compared to the currents induced by ATP at pH 7.5.

Intracellular Ca^{2+} measurements in cultured TG neurons

Primary cultured TG neurons were loaded with Fluo4-AM (3 μM in DMSO, Molecular Probes, Eugene, OR) for 30 min at 37 °C. The cells were then washed twice with PBS and incubated for 30 min at 37°C, and they were mounted in a perfusion chamber that was placed on the stage of a confocal microscope (Zeiss LSM800) at 22–24°C. Purinergic agonists (ATP or α,β-metilATP) were added at the corresponding time at a final concentration of 100 μM in bath solution. Cultured cells were briefly illuminated adjusting laser power (≤ 0,5%) and scan speed (6–7), using the ZEN 2.1 software (Zeiss). Regions of interest were simultaneously selected on neuronal somata containing Fuo4 fluorescence (laser excitation 480 nm, emission 510 nm) in a field having usually more than 20 cells. Images were collected at 2 s intervals during a continuous 12-min period. The imaging was carried out in a 16-bit (0–65000 units of fluorescence scale) with highly sensitive GaAsP detector (Zeiss). The Ca^{2+} transients, defined by their amplitude that should be greater than two times the noise level, were acquired and analyzed off line with ZEN 2.1 software.

Statistical analysis

All experiments were performed a minimum of three times. All graphs show the average ± SEM. Statistical evaluation was performed with GraphPad Prism software, version 6.1 (GraphPad, San Diego, CA). Significant differences between experiments were assessed by an unpaired t test or a one-way ANOVA with a Tukey posttest, considering α as 0.05. For electrophysiological experiments, statistical analyses were performed using the non-parametric Mann-Whitney test.

Results

The P2X2aR binds to the Cdk5/p35 complex in heterologous expression system

To study the potential P2X2aR modulation by Cdk5, we first evaluated whether there is an interaction between the P2X2aR and the Cdk5/p35 complex in HEK293 cells co-transfected with Cdk5, p35, and P2X2aR vectors. For this end, we transfected P2X2aR-GFP fusion vector (Figure 1A), and found that GFP fluorescence from P2X2aR-GFP fusion protein (green) was located mainly on the plasma membrane and it was highly co-localized with P2X2R label (red), allowing us to use GFP fluorescence as a measure of P2X2aR localization (Figure 1B). To confirm the specificity of P2X2R antibody, we used a control peptide antigen for P2X2R that was incubated with P2X2R primary antibody, demonstrating that P2X2R label almost disappears although GFP fluorescence remains (Figure 1C). Next, by immunofluorescence using Cdk5 and p35 antibodies we found that P2X2aR-GFP (green) co-localized with Cdk5 (red) and p35 (blue) immunostaining in close proximity to plasma membrane (Figure 1D). In addition, we performed a set of confocal images at different focal planes (0.9 μm each) (Figure 1E) and an orthogonal reconstruction (Figure 1F) to find evidence that support that P2X2aR-GFP, Cdk5 and p35 are interacting with each other, and they were effectively found co-localized in areas near to the plasma membrane. To confirm the specificity of P2X2R antibody in Western blot analysis, we incubated the P2X2R primary antibody in the presence or absence of control peptide antigen, and we observed that the P2X2R bands disappeared in the presence of the peptide in both heterologous expression of P2X2aR transfected HEK293 cells or in endogenous P2X2R expression in PC12 cells (Figure 2A). We also evaluated whether P2X2aR can immunoprecipitate with Cdk5 or p35 in HEK293 cells co-transfected with P2X2aR-pIRES-GFP with p35 or Cdk5 vectors. By immunofluorescence we observed that P2X2aR expression was localized in plasma membrane and GFP fluorescence was localized in the whole cell (Figure 2B). We performed co-immunoprecipitation experiments using HEK293 cells under the following experimental condition: a) control untransfected cells (UT); b) cells transfected with P2X2aR-pIRES-GFP vector; c) cells transfected with P2X2aR-pIRES-GFP plus p35 vectors; and d) transfected with P2X2aR-pIRES-GFP plus His-Cdk5 vectors, and then we confirmed the expression of transfected proteins through Western blots (Figure 2C). Using protein extracts from HEK293 cells co-transfected with P2X2aR and p35, or from P2X2aR and His-Cdk5, we immunoprecipitated proteins by using an anti-p35 antibody (Figure 2D), or anti-Cdk5 antibody (Figure 2E). In the immuno-complexes from both immunoprecipitates, we detected the P2X2aR in Western blot using an anti-P2X2R antibody. As a control of immunoprecipitation, we detected the corresponding Cdk5 and p35 proteins after stripping each membrane. Moreover, as controls for immunoprecipitation assays we used goat IgG control and no primary antibody (only Protein A/G agarose beads) (Figure 2D and E). These results indicate that P2X2aR form a protein complex with Cdk5 and p35 in co-transfected HEK293 cells. The formation of such complex could be independent of mutual presence of Cdk5 and p35. This finding prompted us to investigate if there is a link between the activities of Cdk5 signaling pathway and P2X2aR.

The P2X2aR is a target for Cdk5 phosphorylation

By using the phosphorylation prediction websites Motif-Scan and NetPhos2.0 we identified a putative consensus motif for Cdk5 phosphorylation of (S/T)PX(K/H/R) in the P2X2aR protein sequence [2]. Remarkably, this consensus motif ^{372}TPKH^{375} sequence is present exclusively in the full-length rat P2X2aR but absent in the shorter splice variant P2X2bR (Figure 3A). By using P2X2R antibody we recognized P2X2aR, P2X2bR, and P2X2aR-T372A protein transfected in HEK293 cells (Figure 3B). We evaluated whether P2X2aR–Cdk5/p35 interactions results in the phosphorylation of threonine residues, an indication that Cdk5 could directly phosphorylate the P2X2aR. To evaluate this, we immunoprecipitated P2X2aR from transfected HEK293 cells with P2X2aR alone or with p35 vector, by using P2X2R antibody followed by Western blot against phospho-threonine antibody. We found that phospho-threonine signal was significantly increased in cells expressing P2X2aR and p35, as compared with P2X2aR alone (Figure 3C). To further study whether this P2X2aR sequence is a phosphorylation target for Cdk5/p35, we generated 10 amino acid peptides containing the threonine Cdk5 motif present in rat, mouse and human P2X2aRs (Figure 3D) and tested their phosphorylation potential by an *in vitro* kinase assay by using recombinant Cdk5/p35 protein. As a positive control, we used the phosphorylation of Histone H1 as measured by the incorporation of ^{32}P. We found that all three P2X2aR-derived peptides were highly phosphorylated by the recombinant Cdk5/p35 complex with 45.9% for human P2X2aR, 56.3% for mouse P2X2aR, and 64.0% for rat P2X2aR as compared with Histone H1 phosphorylation (100%). A non-related 10 amino acid peptide was used as negative control for Cdk5 activity and it was one order of magnitude less phosphorylated (3,4%) than P2X2aR peptides (Figure 3D). These experiments also showed that *in vitro* phosphorylation of P2X2aR-derived peptides from human, rat and mouse occurred at the same extent when using similar experimental conditions.

Cdk5-activation regulates P2X2aR desensitization

We tested if this interaction between the P2X2aR and Cdk5/p35 has any functional effects. For this purpose, we studied the patterns of currents generated by P2X2aR activation using electrophysiological techniques and tested Cdk5-mediated changes in those currents. As we have previously demonstrated, the P2X2R has a unique property, an increase in receptor desensitization rate after repetitive ATP applications that is termed use-dependent desensitization (UDD) [14; 27]. In HEK293 cells expressing the P2X2aR and measured under the whole-cell configuration, receptor desensitization increased after repetitive 10 μM ATP applications in the presence of extracellular Ca^{2+} (Figure 4A). Cells co-expressing the P2X2aR and p35 still showed UDD although the percentage of desensitization in the third and fourth ATP application was significantly smaller (Figure 4A and *C*, n=6, $p<0.05$). In contrast, no difference in the development of UDD was found between cells expressing the P2X2bR alone or co-expressed with p35 (Figure 4B and *D*, n=6). Activation of Cdk5 by transfection of p35 did not affect the current amplitudes of both receptors (Figure 4E and F). Afterwards, we wanted to test these effects in *Xenopus laevis* oocytes, a different heterologous expression system. The increase in receptor desensitization in oocytes was significantly lower, and only after four 100 μM ATP applications a modest increase in receptor desensitization was observed (Figure 5A and B). However, co-expression P2X2aR and p35 prevented the modest increase in desensitization observed at the fourth 100 μM ATP

application (Figure 5A, B and E), suggesting a role of Cdk5 in this effect. No differences in ATP EC_{50} (Figure 5C) nor in maximal current (Figure 5D) were observed when the P2X2aR was expressed alone or together with p35. Finally, the the treatment with the Cdk5 inhibitor roscovitine (30 μM, incubated for 3 h before the experiment) reverted the effect of Cdk5 activation and restored the desensitization observed in the P2X2aR alone (Figure 5E). Thus, Cdk5-mediated phosphorylation should be responsible for P2X2aR desensitization.

In order to disrupt the putative Cdk5 phosphorylation site of the P2X2aR, ^{372}TPKH^{375}, we generated the P2X2aR-T372A mutant. This mutant was successfully expressed in the plasma membrane of HEK293 cells and was indistinguishable from wild-type P2X2aR, as measured by immunofluorescence experiment (Figure 6A and B). In addition, this mutant has similar molecular weight as compared with wild-type P2X2aR (Figure 3B). In functional experiments, the whole-cell current elicited by ATP desensitized with an average τ_{des} of 10.3±1.2 s, but no further increases in UDD were observed as compared to the wild-type P2X2aR in HEK293 transfected cells (Figure 6C). Next, we overexpressed both the wild-type P2X2aR and the T372A mutant in PC12 cells during 24 h and we observed similar results to that obtained with HEK293 cells (Figure 6D). Similar to what we observed for wild-type P2X2aR, when the T372A mutant was expressed in oocytes the rates of receptor desensitization were slower than in HEK293 cells (Figure 6E). In addition, ATP affinity was not significantly affected by this mutation (14.6±2.5 μM in T372A vs. 17.7±0.7 μM in wild-type P2X2aR, Figure 6F), and the maximal currents were affected only in oocytes but not in HEK293 cells (Figure 6G). We hypothesize that the mutation induces important structural changes that affects the receptor gating, rendering a less-desensitizing phenotype and preventing the effect of Cdk5 activation as observed in wild-type receptors. To confirm that the T372A mutation did not alter other features of P2X2aR gating, we studied the effects of several allosteric modulators and one antagonist of this receptor. The typical potentiation of ATP-gated currents of P2X2aR induced by copper, zinc and protons was conserved in the T372A mutant as well as the inhibition induced by PPADS (Figure 6H). Finally, co-expression of the T372A mutant and p35 did not affect receptor desensitization in both HEK293 cells and oocytes (Figure 6H). Thus, these results suggest that Cdk5 mediated phosphorylation of the P2X2aR at Thr372 can mainly affect the desensitization of this receptor channel.

P2X2R interacts with Cdk5/p35 complex in nociceptive mouse tissues

To evaluate whether P2X2R is also interacting with Cdk5 and p35 in relevant physiological tissues, we performed primary cultures of neurons from mouse DRG and TG. After 2 days of culture, by using immunofluorescence and confocal microscopy we detected that P2X2R (green) is expressed exclusively in a subset of TG (Figure 7A and C) and DRG (Figure 7B and D) neurons co-localizing with p35 (arrows, Figure 7A and *B*) or Cdk5 (arrows, Figure 7C and *D*). The expression of Cdk5 and p35 (red) was predominant in neurons, labeled with βIII-tubulin or Map1B antibodies (white). Cdk5 and p35 were also detected in others cell types present in our primary culture setup although showing reduced relative signal intensity (Figure 7A–D). We also detected co-localization of P2X2R with p35 (Figure 7E) or P2X2R with Cdk5 (Figure 7F) in neurites from primary culture TG neurons. Moreover, by using RT-PCR we detected that mouse TG and DRG expressed mRNA of P2X2R isoforms (P2X2aR,

P2X2bR, and P2X2eR), P2X3R, Cdk5 and p35 (Figure 7G). In addition, by using Western blot we detected several bands of P2X2R (between 55 and 72 kDa) from mouse TG and DRG tissues, according to splice variants and a different degree of glycosylation [39]. We also detected P2X3R, Cdk5 and p35 by western blot from these tissues (Figure 7H). Finally, we immunoprecipitated protein extracts from mouse TG by using a rabbit anti-Cdk5 antibody and detected P2X2R in Western blot using a rabbit anti-P2X2R antibody. As a control for immunoprecipitation, we detected Cdk5 protein after stripping the membrane. As a control with no primary antibody, we only used Protein A/G agarose beads and we did not find P2X2R in those immuno-complexes (Figure 7I). Altogether, these results indicate that endogen P2X2R interacts with Cdk5 in mouse TG and that P2X2R co-localize with Cdk5 and p35 in TG and DRG neurons, suggesting that Cdk5 might play an important role in physiological modulation of P2X2R, implicating its role in pain signaling.

Endogenous P2X2R-mediated currents are regulated by endogenous Cdk5 activity

In a following set of experiments, we tested whether Cdk5 regulation could be relevant in a system that expresses both proteins endogenously by means of electrophysiology. We chose for this purpose neuronal-like PC12 cells, a cell line derived from rat pheochromocytoma that was the original source for the first P2X2R cloned [4] (Figure 2A). These cells also endogenously express p35 and Cdk5 [23; 53]. Undifferentiated PC12 cells exhibit ATP-mediated currents that match with the desensitization profile of the slow desensitizing P2X2aR (τ_{des}=22.9±3.4 s; Figure 8A and *B*). When cells were treated with roscovitine (30 μM) for 6 or 24 h in order to inhibit the endogenous Cdk5 activity, currents desensitized significantly faster (τ_{des} 6h=7.1±2.5 s; τ_{des} 24h=9.0±2.0 s; Figure 8A and *B*), without significantly altering the current densities (Figure 8C), further confirming the role of Cdk5 in regulating the gating properties of the P2X2aR.

The desensitization of the heteromeric P2X2/3R is also modulated by Cdk5/p35

In a final set of experiments we tested the effects of Cdk5 on heteromeric P2X2/3Rs, which is the main purinergic subtype, expressed in pain pathways. In HEK293 cells co-transfected with the P2X2aR and the P2X3R, we evoked heteromeric currents by using the specific agonist α,β-meATP that induces the typical slow desensitizing P2X2/3R-mediated currents (Figure 8D); when p35 was co-transfected with both P2XRs the resulting current desensitized slower than the one mediated by the P2X2/3R alone, with desensitization constants of 10.7±1.9 and 5.8±0.8 s, respectively (Figure 8D and E), without significantly altering the current densities (Figure 8F). To test the physiological relevance of this phenomenon we first performed primary cultures of mouse TG (Figure 9A) and DRG neurons (Figure 9B) for 2 days and we observed co-localization of P2X2R (red) with P2X3R (green) by immunofluorescence. Later on, in acute primary cultures of rat TG we measured the endogenous P2X2/3R mediated responses by means of Ca^{2+} imaging. ATP evoked strong responses of TG neurons, as reflected by the increases in fluorescence induced by ATP in Fluo-4 loaded neurons (Figure 9C). ATP, a general purinergic agonist, activated in average a 30% of the stimulated neurons (Figure 9D). Interestingly, when α,β-meATP was used, a P2X3R and P2X2/3R agonist, only an 8% of the TG neurons responded (Figure 9D). The responding cells presented two main types of responses, a fast spike (black tracing), probably reflecting the activation of a homomeric P2X3R, and a slower spike (red

tracing), that could reflect the activation of the P2X2/3R (Figure 9E). Finally, when TG neurons were treated with roscovitine (20 μM, 6h), we observed a greater number of fast spikes responses (Figure 9F), that resulted in a decrease of the decay constant (Figure 9G) without affecting the percentage of responding cells (Figure 9H) nor the relative amplitude (Figure 9I) of the α,β-meATP evoked responses.

Discussion

In the present study we showed a functional interaction between the P2X2aR and the Cdk5/p35 complex. Moreover, the currents mediated by this channel are regulated by the activation of Cdk5, which prevents the development of UDD in whole-cell patch-clamp recordings. We suggest that the phosphorylation of the intracellular P2X2aR residue Thr372 by Cdk5/p35 delays the receptor desensitization and such modulation can be physiologically important, especially in pain signaling pathways where both proteins are interacting, as our experiments in PC12 cells and TG neurons strongly suggest.

Phosphorylation and dephosphorylation are common processes that regulate diverse protein functions, including the gating of plasma membrane channels; a number of reviews have summarized the effects of phosphorylation in voltage-gated channels as well as in NMDA, GABA, TRP and ACh receptors [10; 37; 45; 50]. P2XRs gating is also modulated by phosphorylation and dephosphorylation. For example, it has been suggested that PKA regulates P2X2R and P2X3R function, inhibiting the currents mediated by the former and potentiating the latter [16; 59]. In the case of the P2X4R, PKA seems to regulate the receptor trafficking through an accessory protein [6]. PKC has also been suggested as a regulator of P2XR-activity, an effect that for the P2X1R can be mediated by the activation of G-protein coupled receptors [1]. Additionally there is evidence suggesting an effect of PKC on P2X3R [43]. A recent study has shown that calmodulin kinase II can regulate the P2X3R trafficking from and to the plasma membrane [15]. Src tyrosine kinase has also been suggested to regulate the P2X3R, decreasing the receptor-mediated currents through the intracellular Tyr393 [17].

Cdk5 regulates TRPV1 channel function by direct phosphorylation at Thr407 increasing the Ca^{2+}-influx mediated by this channel [25; 33; 40; 47], and by phosphorylation of kinesin 13B (KIF13B) motor that increases TRPV1 trafficking to the plasma membrane [61]. In this context, the results showed here are in agreement with previous Cdk5 mechanism of action in the pathways of pain signaling [52]. Thus, Cdk5-mediated P2X2aR phosphorylation on Thr372 will modify the degree of desensitization of the channels that will finally result in an increased depolarization and increased of Ca^{2+} influx in the nociceptive neurons. Contrary to TRPV1 and P2X2aR, Cdk5 activation seems to downregulate P2X3R function through the serine phosphorylation of an unidentified accessory protein [38]. This could reflect a differential regulation of homomeric P2X3Rs and heteromeric P2X2/3Rs, that are the subtypes mainly expressed in nociceptive neurons; future functional experiments in nociceptive DRG or TG neuron will help to clarify the role of Cdk5 regulation in these two P2XR channel subtypes.

In the present study, our results support a direct interaction of the P2X2aR with Cdk5 and p35. Co-immunolocalization experiments showed a marked overlap between P2X2aR and Cdk5/p35 signals in both heterologous systems and endogenous protein levels as we found in mouse TG and DRG neurons; this was further supported by co-immunoprecipitation experiments, suggesting that P2X2aR can interact with both Cdk5 and p35. Additionally, we also found that P2X2aR and Cdk5/p35 not only are physically close, but also that their interaction increases threonine phosphorylation. Moreover, the Cdk5 consensus motif ^{372}TPKH^{375} is only present in the rat P2X2aR but not in the splice variant P2X2bR. We identified this sequence as part of the canonical consensus sequence (S/T)PX(K/H/R) present in target proteins that are phosphorylated by Cdk5 [2] and we confirmed that this sequence can be a phosphorylation target by the synthesis of peptides containing this sequence and the homologous Cdk5 consensus motifs present in human, rat and mouse P2X2aRs; all of these peptides were phosphorylated by the Cdk5/p35 complex, indicating that they can effectively reflect a phosphorylation site in the P2X2aR C-terminal domain.

We tested if the Cdk5 mediated phosphorylation of the P2X2aR could have functional consequences in the activity of this channel, by means of electrophysiological recordings. We found that the activation of endogenous Cdk5, by the expression of its activator p35, significantly prevented P2X2aR desensitization after repetitive ATP applications. We have previously shown that the P2X2aR increases its desensitization rates after successive ATP applications, arising a Ca^{2+}-dependent process termed UDD [14; 27]. The most plausible explanation for this phenomenon is that Cdk5-phosphorylation favors a non-desensitized phenotype and protects the receptor for the Ca^{2+}-dependent UDD. We have previously showed that UDD requires the presence of Ca^{2+} in a nanomolar or less concentration to be observed in whole-cell recordings [14] and this could reflect the loss of some relevant cytosolic factor to determine P2X2aR desensitization. This can explain the differences observed in the recordings performed in HEK293 cells and *Xenopus* oocytes, though future experiments will help to completely clarify the mechanism of P2X2aR desensitization. When experiments were performed in *Xenopus* oocytes, a recording system that preserves the intracellular components, we also observed a prevention of P2X2aR increase in desensitization after Cdk5 activation and this effect was blocked by the Cdk5-inhibitor roscovitine, although desensitization was remarkably smaller than in whole-cell patch-clamp recordings. Moreover, the mutation of T372A prevented the development of UDD in whole-cell recordings in HEK293 cells and inhibited the increase of desensitization in two-electrode voltage-clamp recordings in oocytes suggesting the critical role of this residue in this phenomenon. Although we initially expected that this mutant would render more desensitized profiles of currents we hypothesize that the mutation induced a structural change that probably resulted in this particular phenotype. Although the T372A mutation obliterated the increases in receptor desensitization regardless of the expression system used is it obvious that the desensitization observed varied depending on the cell host, showing a higher desensitization profile in HEK293 cells as compared to PC12 cells and *Xenopus* oocytes. We infer that these differences could be the result of particular features of each expression system, but in despite of that, we confirm that the mutant T372A preserved most of the features characteristic of the P2X2aR, supporting our findings in the present work.

Remarkably, the experiments in PC12 cells and in primary cultures of TG neurons strongly suggest that Cdk5-mediated regulation of the P2X2R could be physiologically relevant. PC12 cells endogenously express P2X2R, Cdk5 and p35 [4; 23; 53]; we inferred that if that being the case, the P2X2R-mediated currents are basally phosphorylated by Cdk5, rendering currents with slow desensitization kinetics. As expected, Cdk5 inhibition by roscovitine accelerated the desensitization of P2X2R-mediated currents, in agreement with the results obtained in heterologous systems. Finally, we studied the effects of Cdk5 phosphorylation on the heteromeric P2X2/3R that is the main purinergic receptor involved in pain transmission [31]. Currently, there is no published information if all the splice P2X2R variants can indeed form heteromeric receptors, but we have preliminary data that at least rat P2X2bR is able to form functional heteromeric channels (Stojilkovic, personal communication). As the homomeric P2X2aR, the desensitization properties of the P2X2/3R were affected by Cdk5 phosphorylation as demonstrated by both electrophysiology of heterologous expressed P2X2/3Rs in HEK293 cells and by Ca^{2+} imaging of endogenous P2X2/3Rs from primary cultures of TG neurons that also express endogenously p35 and Cdk5. These results strongly suggest a physiological relevance of Cdk5 regulation of purinergic receptors, especially in pain transduction. P2X2/3Rs are commonly formed by two P2X3 subunits and one P2X2 subunit [26]; however a recent paper has shown that the $P2X3(P2X2)_2$ stoichiometry also results in functional channels [29], we infer that these receptors will be even more susceptible to Cdk5 regulation.

In general, the effects of phosphorylation on membrane receptors and channels can be exerted through direct regulation of the channel gating properties resulting in a greater or smaller conductance or in a change of voltage dependence or in the agonist's EC_{50}; as it has been observed for Kv2.1 or TRPV1 channels [36; 42]. Other described effects of channel phosphorylation o dephosphorylation are channel internalization and/or changes in trafficking and degradation as for example AMPA and NMDA glutamate receptors [24; 32]. Interestingly it has also been reported that a phosphorylation and dephosphorylation can regulate the desensitization of NMDA receptors, by regulating the number of functional channels in the membrane [58]. In our study, it seems that Cdk5 phosphorylation does not affect the number of functional channels (it has no effect on current amplitudes) but rather it affects the desensitization properties by changing its gating properties, because we did not observe changes in the maximal currents nor in ATP EC_{50} after Cdk5 activation. Accordingly, we did not observe a significant increase in the protein levels of the P2X2aR in immunoblots when the receptor was co-expressed with p35 or Cdk5, however future studies will be conducted to explore P2X2aR trafficking to the plasma membrane when Cdk5 is highly activated. There is evidence showing that Cdk5 regulate the activation of several ion channels and membrane receptors, suggesting a role during peripheral and central sensitization in pain pathways [21; 47; 52; 60]: For example Cdk5 can phosphorylate NMDA receptors at Ser1232 of the NR2A subunit [32] and regulate its surface expression through NR2B subunit phosphorylation [44]. P/Q-type voltage-dependent Ca^{2+} channels are also targets for Cdk5 phosphorylation, a process that regulates neurotransmitter release [51].

In summary, we have identified a novel mechanism of regulation of P2X2R desensitization by Cdk5-mediated phosphorylation, and we have presented evidence that this regulation could be relevant in pain signaling, such as P2X2/3R mediated responses in TG neurons.

Future efforts should be focused on characterizing the mechanism of P2X2aR desensitization and whether phosphorylation and Ca^{2+}-mediated effects are related. Our findings suggest that the P2X2aR is a novel substrate for Cdk5-mediated phosphorylation, which might play an important role in the physiological processes such as pain signaling and therefore could be a potential novel target for developing pain therapies.

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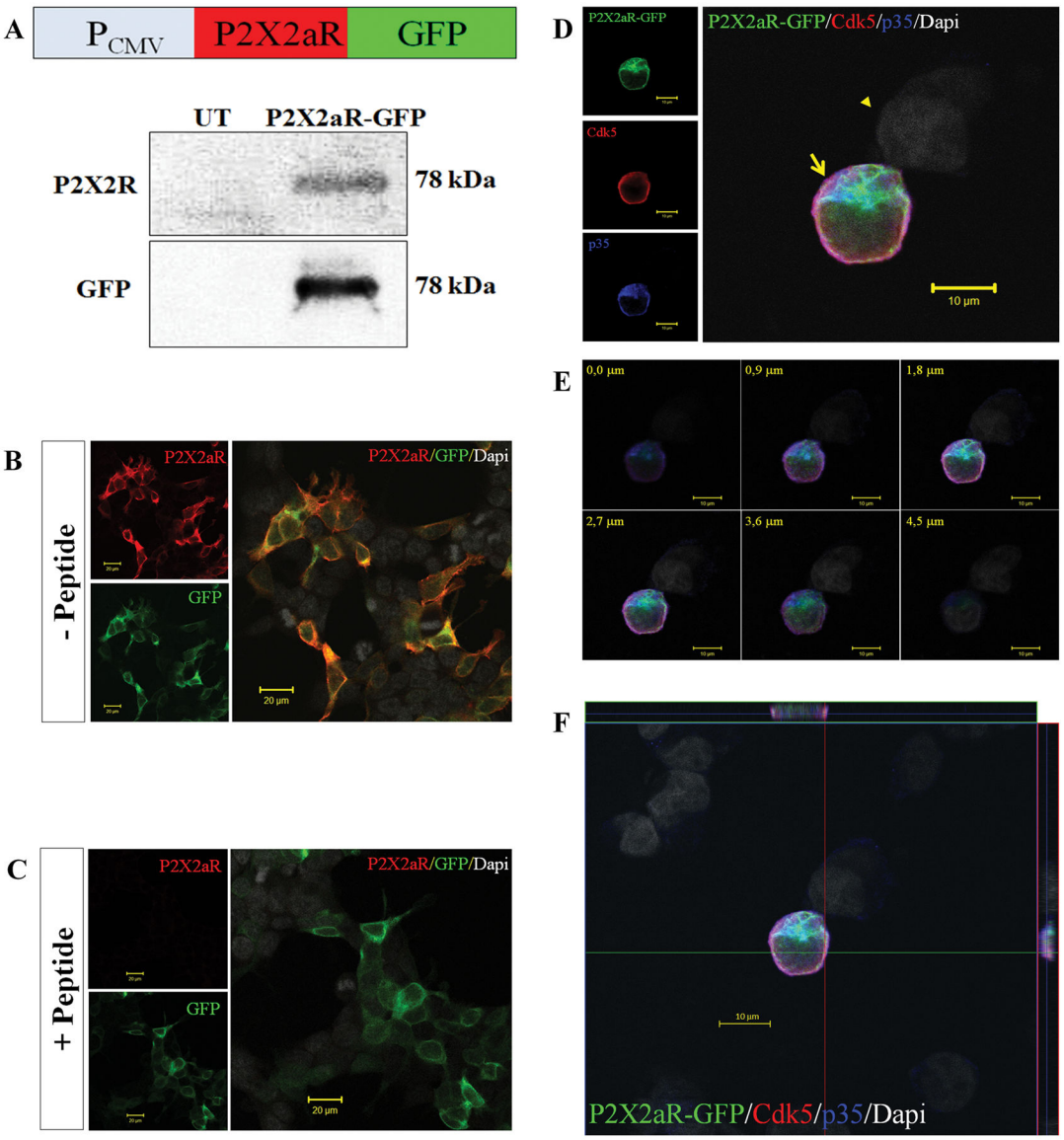

Figure 1. Co-localization of P2X2aR with Cdk5/p35 in HEK293 cells

A. Upper panel, scheme of P2X2aR-GFP vector used for transfection of HEK293 cells. Lower panel, Western blot for P2X2R and GFP from HEK293 cells transfected with P2X2aR-GFP vector or untransfected (UT). **B** and **C**. Confocal images of HEK293 cells expressing P2X2aR-GFP vector in absence (**B**) or in presence (**C**) of control peptide antigen for P2X2R. GFP fluorescence from P2X2aR-GFP fusion protein (green) was located mainly in the plasma membrane and it highly co-localizes with P2X2R label (red). Dapi (white) was used for nuclear staining. **D.** Confocal immunofluorescence of HEK293 cells expressing P2X2aR-GFP, Cdk5 and p35 vectors (arrow) and untransfected HEK293 (head arrow). P2X2aR-GFP green fluorescence was observed co-localizing with Cdk5 (red) and p35 (blue) immunostaining, both close to plasma membrane. **E.** Set of six confocal images at different focal planes (0.9 µm each) showing co-localization of P2X2aR-GFP with Cdk5/p35. **F.**

Orthogonal analysis reveals a high co-localization of P2X2aR-GFP, Cdk5, and p35 in region near to plasma membrane of triple transfected HEK293 cells.

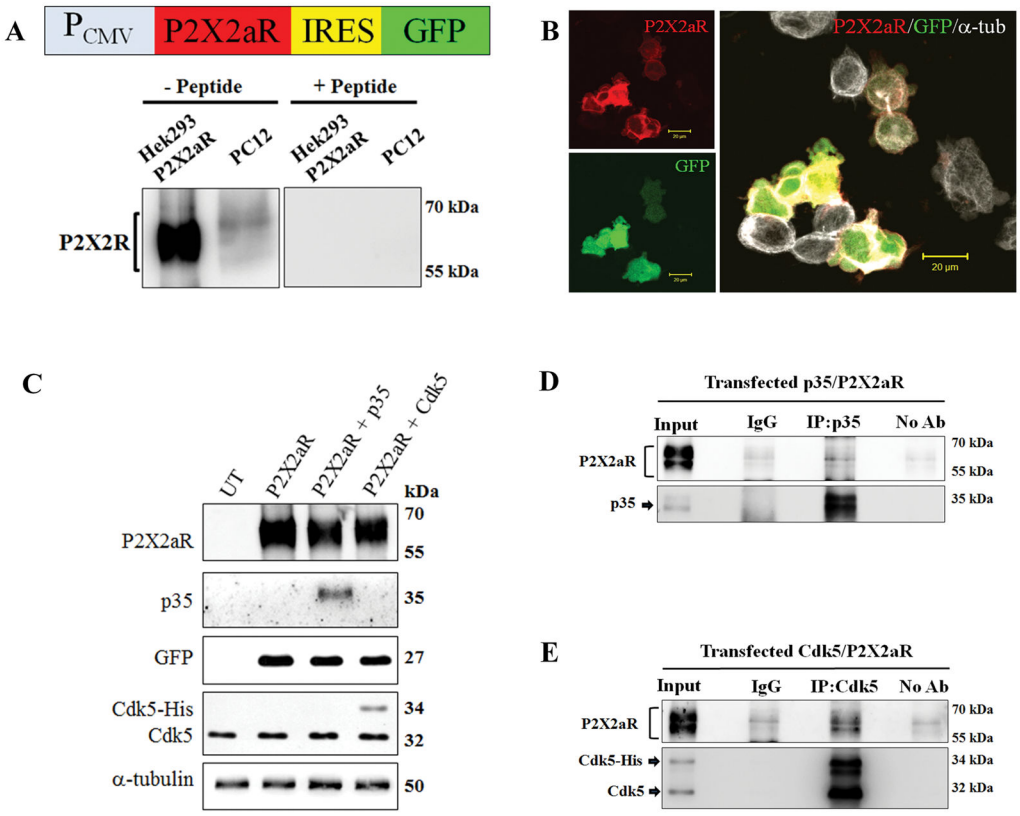

Figure 2. Co-immunoprecipitation of P2X2aR with Cdk5/p35 in HEK293 cells

A. Upper panel, scheme of P2X2aR-IRES-GFP vector used for transfection of HEK293 cells. Lower panel, Western blot for P2X2R from HEK293 transfected or from PC12 in the absence or the presence of control peptide antigen for P2X2R. **B**. Confocal images of HEK293 cells expressing P2X2aR-IRES-GFP vector. GFP fluorescence (green) was located in entire transfected cells; in contrast P2X2aR immunostaining was mainly localized in the plasma membrane. α-tubulin (white) was used as cellular staining. **C.** Analysis of protein expression of HEK293 cells expressing P2X2aR-pIRES-GFP, His-Cdk5, and p35 vectors. **D** and **E**. Immunoprecipitation experiments of HEK293 cells expressing P2X2aR-pIRES-GFP and p35; or P2X2aR-pIRES-GFP and Cdk5. For immunoprecipitation we used p35 rabbit (C19) antibody (**D**) and Cdk5 mouse (DC17) antibody (**E**) and we detected P2X2aR by using P2X2R rabbit antibody by Western blot. As a positive control, the corresponding Cdk5 and p35 proteins were detected after stripping of each membrane. For a control with no antibody, we only used Protein A/G agarose beads and we did not find the P2X2aR in those immunocomplexes. We used normal IgG goat antibody as IgG control.

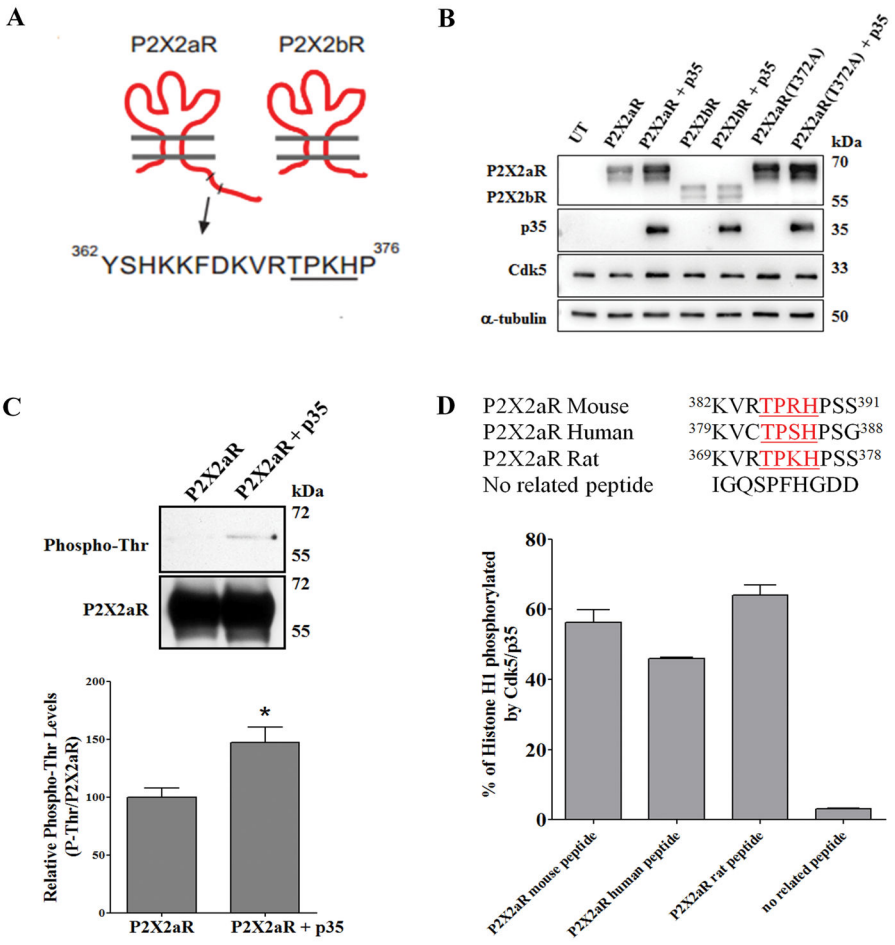

Figure 3. Cdk5/p35 phosphorylates the P2X2aR

A. Representative cartoon showing the difference between the full-length P2X2aR and the short-variant P2X2bR, highlighting the putative Cdk5 site on the P2X2aR. **B.** Representative Western blots against P2X2R, p35, and Cdk5 of HEK293 cells transfected with P2X2aR, P2X2bR, and P2X2aR-T372A, in the presence or absence of p35. α-tubulin is a loading control. **C.** Upper panel, representative immunoprecipitation experiment on HEK293 cells transfected with P2X2aR-pIRES-GFP alone or with p35. Cell lysates were immunoprecipitated with a P2X2R antibody and then revealed with a Phospho-Thr antibody that recognizes phosphorylation in Cdk5-protein targets. Lower panel, Quantification of the increase in Phospho-Thr levels in cells co-expressing P2X2aR and p35, calculated from a densitometry analysis. n=3, $p<0.05$ t-Test. **D.** Upper panel, sequences of the synthetic peptides derived from P2X2aR from mouse, human and rat that include a specific underlined Cdk5 consensus motif (red) and a sequence of a non-related peptide as negative control. Lower panel, ***in vitro*** phosphorylation mediated by Cdk5/p35 complex detected by ^{32}P incorporation into the synthetic peptides. Histone H1 was used as positive control of Cdk5/p35 kinase activity and a non-related peptide was used as a negative control. n=6 measures for each reaction.

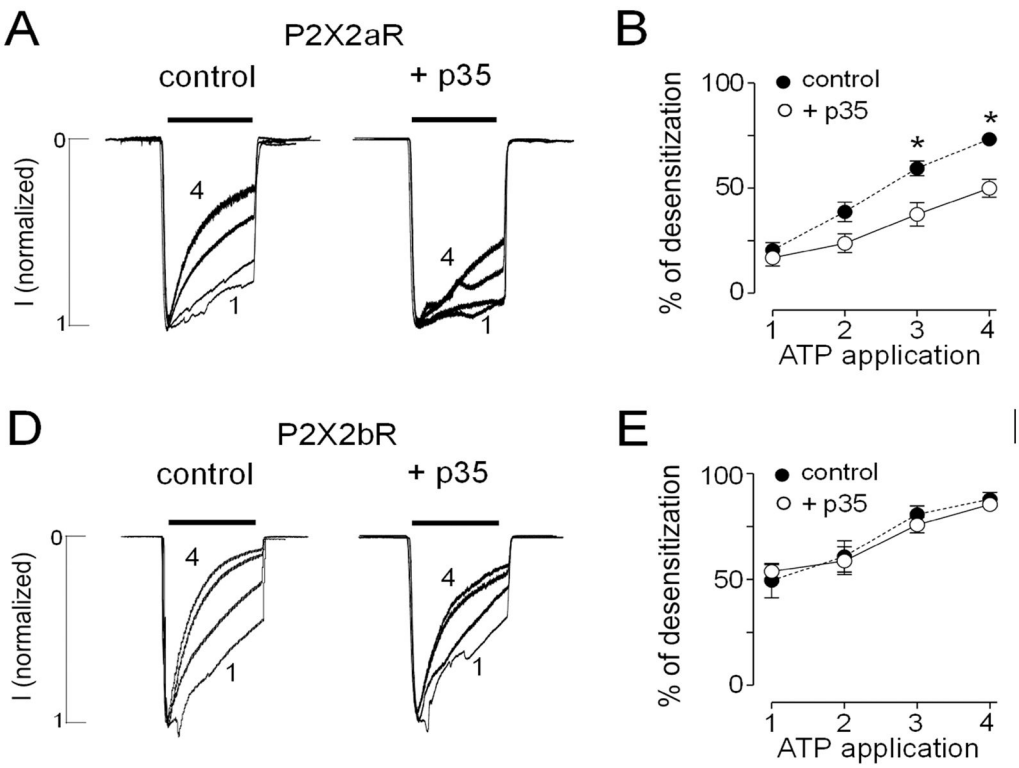

Figure 4. Cdk5 activation delays P2X2aR desensitization expressed in HEK293 cells

A and **D.** Representative recordings of consecutive 10 μM ATP applications in the presence of extracellular Ca^{2+}, to single cells expressing the P2X2aR (**A**) or the P2X2bR (**D**) alone (left recordings) or co-expressed with p35 (right recordings). **B** and **E**. Summary of the percentages of desensitization in cells expressing the P2X2aR (**B**) or the P2X2bR (**E**) alone (black circles) or co-expressed with p35 (open circles). * $p < 0.05$, Man-Whitney test, n=6. **C** and **F**. Current amplitudes evoked by 10 μM ATP in HEK293 cells expressing the P2X2aR (**C**) or P2X2bR (**F**) alone or co-expressed with p35. n=5.

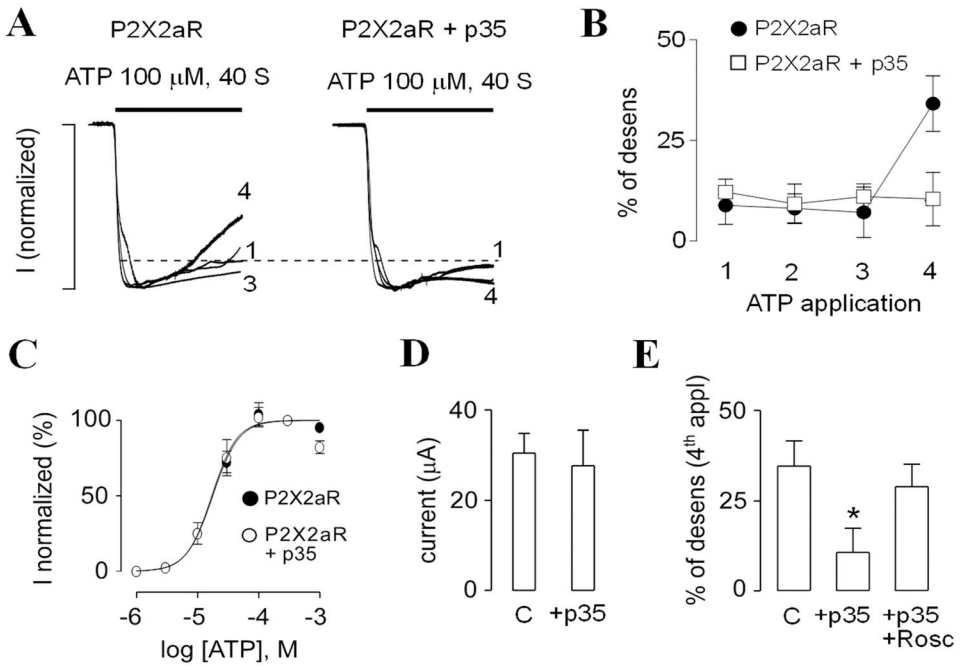

Figure 5. Effects of Cdk5 activation on the P2X2aR expressed in *Xenopus* oocytes

A. Representative recordings of an oocyte expressing the P2X2aR alone (left recordings) or and oocyte co-expressing the receptor with p35 (right recordings). Oocytes were challenged with 100 μM ATP and the first 40 s of applications are shown. In each case, 4 applications with 12 min washouts are shown. **B.** Summary of the percentages of desensitization after four consecutive 100 μM ATP applications. *p<0.05, Mann-Whitney test; n=5. **C.** Concentration-response curves for the P2X2aR alone (closed circles) or co-expressed with p35 (open circles). The estimated EC_{50}s were 17.7 ± 0.7 (●) and 17.1 ± 1.9 (o) μM, n=5. **D.** Summary of current amplitude from oocytes expressing the P2X2aR alone (c) or together with p35 (+p35); n=7. **E.** Roscovitine (Rosc) treatment recovers the percentage of desensitization at the fourth ATP application observed in control oocytes (c) expressing the P2X2aR alone, n=4–5. *p<0.05, Mann-Whitney test.

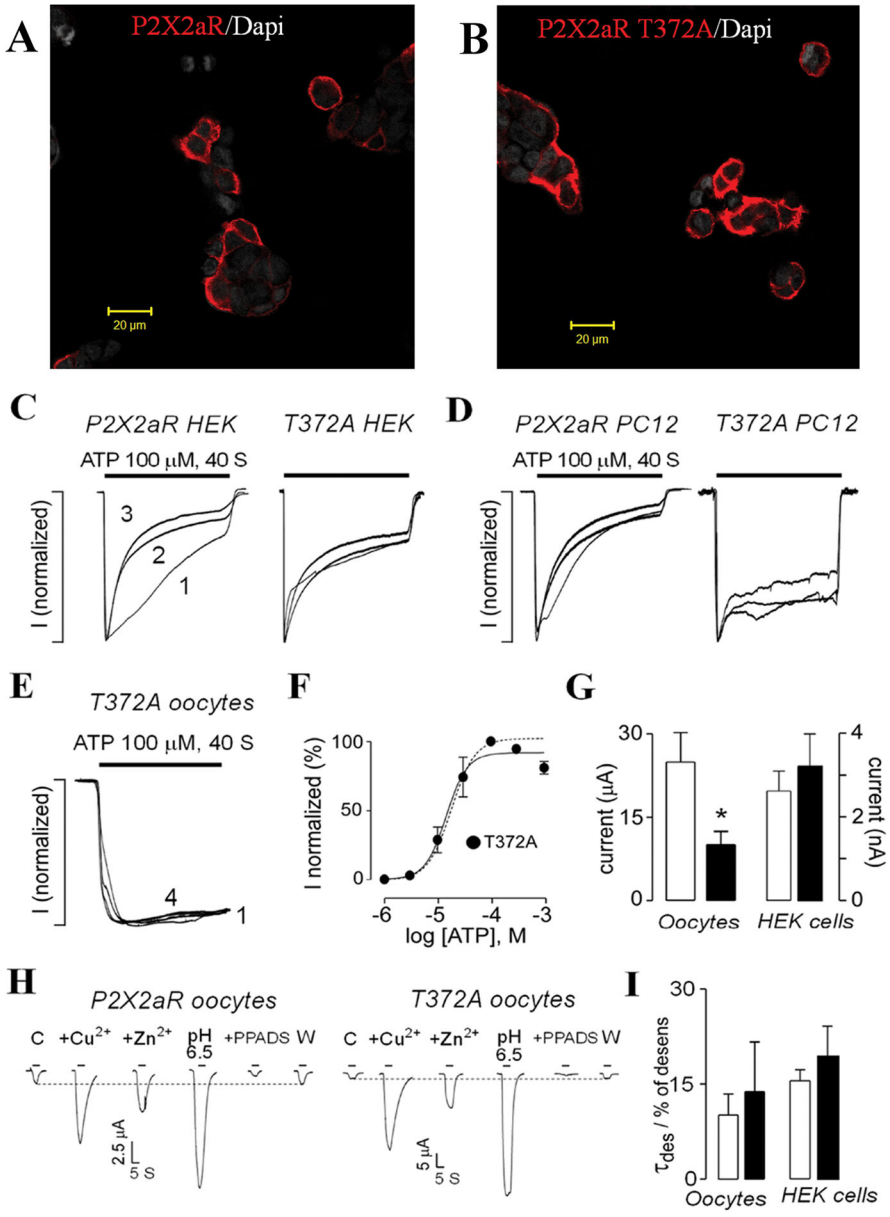

Figure 6. The mutant P2X2aR-T372A loses Ca^{2+}-induced increase in desensitization

Immunofluorescence of HEK293 cells expressing the wild-type P2X2aR (**A**) or the T372A mutant (**B**) in red. Dapi (white) was used as nuclear staining. **C.** Recordings from a HEK293 cell overexpressing the wild-type P2X2aR (left recording), showing the increase in desensitization after four consecutive 100 µM ATP applications, or the T132A mutant (right recordings) showing no further increase in desensitization after repetitive ATP applications. **D.** Recordings from a PC12 cell overexpressing the wild-type P2X2aR (left recordings) or the T132A mutant showing similar results to that obtained with HEK293 cells. **E.** The same protocol in an oocyte expressing the T372A mutant and recorded with two-electrode voltage-clamp technique. **F.** ATP concentration-response curve for the T372A mutant expressed in *Xenopus* oocytes. Dotted line represents the curve for the wild-type P2X2aR.

n=4. **G.** Maximal currents for the wild-type P2X2aR (open bars) and the T372A mutant (closed bars) obtained in oocytes and HEK293 cells. * $p<0.05$, Man-Whitney test, n=5. **H.** Allosteric modulation and inhibition of P2X2aR-mediated currents for the wild-type P2X2aR (left recordings) and the T132A mutant (right recordings). 10 μM of copper (Cu^{2+}), zinc (Zn^{2+}) or PPADS were pre-applied for 1 min before its co-application with 10 μM ATP; C=control (ATP alone), W=washout. Recordings are representative of 4 separate experiments. **I.** Desensitization constants (τ_{des}) and percentage of desensitization (% of desens) calculated for HEK293 cells and oocytes respectively expressing the T372A mutant alone (open bars) or co-expressed with the p35 (closed bars) ATP application; no significant differences in desensitization were observed, n=4–8.

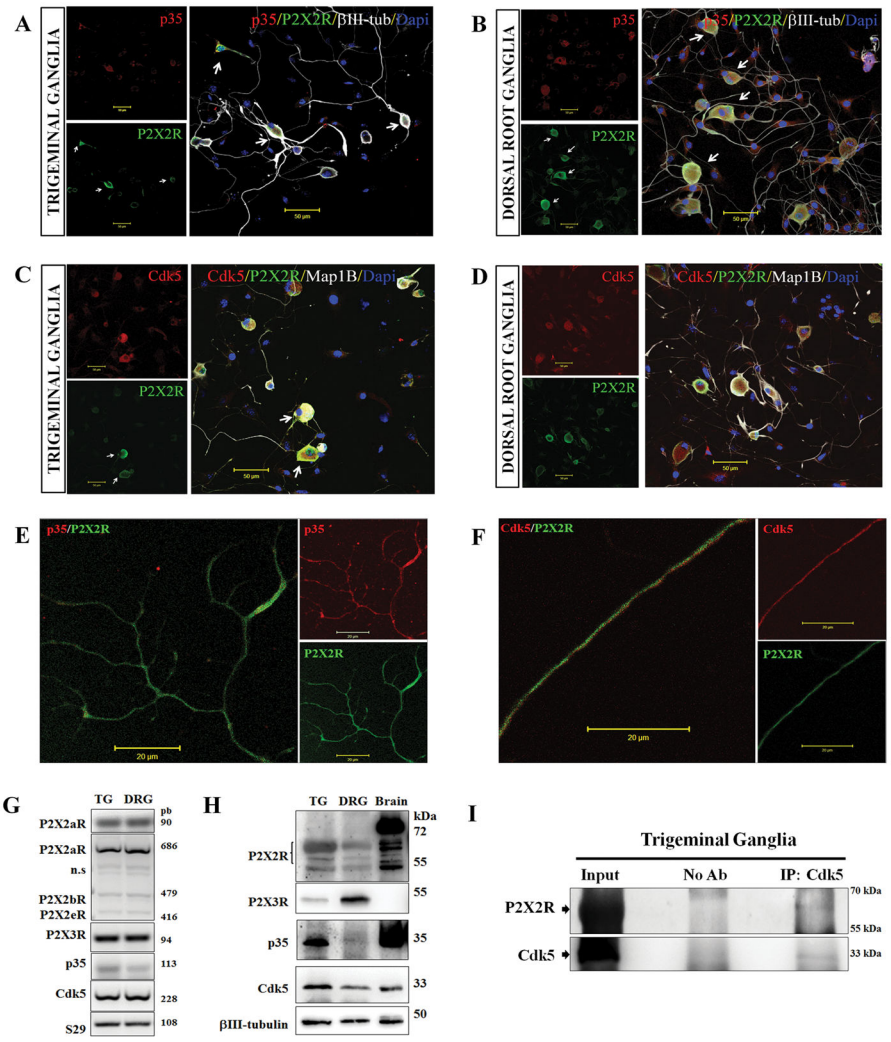

Figure 7. Interaction of P2X2R with Cdk5/p35 in mouse nociceptive neurons

Confocal immunofluorescence of primary cultured neurons of 2 DIV from mouse TG (**A** and **C**) and DRG (**B** and **D**), expressing endogenous levels of P2X2R (green), p35 and Cdk5 (Red). Neurons were immunostained with βIII-tubulin or Map1B (white). Arrows show TG or DRG neurons positive for P2X2R staining. **E.** Magnification of representative neurites from cultured TG neurons stained with P2X2R (green) and p35 (red). **F.** Magnification of representative neurites from cultured TG neurons stained with P2X2R (green) and Cdk5 (red). **G.** Representative RT-PCR from total RNA obtained from mouse TG and DRG tissues. P2X2aR, P2X2bR, P2X2eR, P2X3R, p35 and Cdk5 mRNA levels were evaluated. S29 levels were used as housekeeping control. **H.** Representative Western blots of protein extract from mouse TG, DRG, and brain tissues showing P2X2R, P2X3R, p35, Cdk5 and βIII-tubulin expression. **I.** Immunoprecipitation experiments of mouse TG protein extracts with Cdk5 antibody and detected by Western blot for P2X2R. As a positive control, the corresponding Cdk5 bands were detected after stripping the membrane. For a control with no antibody, we only used Protein A/G agarose beads and we did not find the P2X2R in those immunocomplexes.

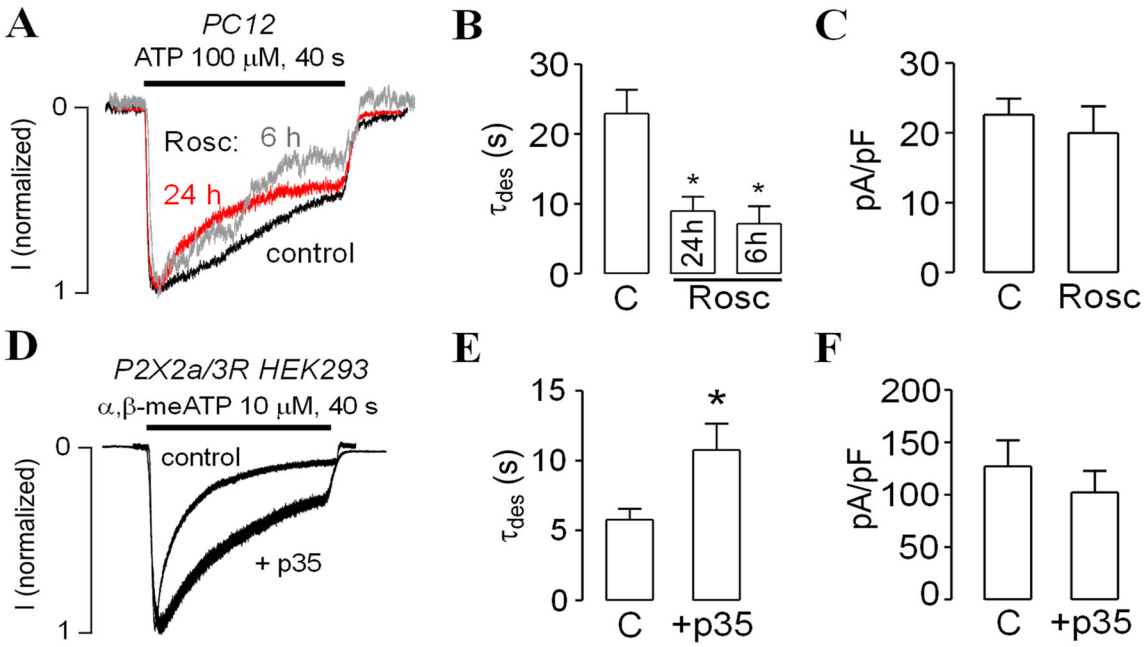

Figure 8. Activation or inhibition of Cdk5 activity regulates P2X2aR-mediated currents in cell lines

A. Representative recordings evoked by 100 μM ATP on PC12 cells in the absence (black tracing), after 6 h (gray tracing) or 24 h (red tracing) incubation with 30 μM roscovitine. Membrane voltage was held at −60 mV. **B.** Summary of desensitization constants (τ_{des}) calculated for PC12 cells in control conditions (C) and after 6 and 24 h of incubation with roscovitine (Rosc). * $p<0.05$, Man-Whitney test, n=5. **C.** Summary of current densities obtained in control (C) and roscovitine-treated (Rosc) PC12 cells. n=5. **D.** Representative recordings of a HEK293 cell expressing the P2X2/3R alone or co-transfected with p35 and the typical slow-desensitizing current (as compared to the fast-desensitizing P2X3R-mediated current) evoked by 10 μM α,β-meATP. **E.** Summary of the desensitization constants (τ_{des}) of the currents mediated by the P2X2/3R alone (C) or co-transfected with p35 (+p35). n=4–8; *$p<0.05$, Mann-Whitney test. **F.** Summary of current densities obtained in the absence (C) and in the presence of p35 (+p35) in P2X2a/3R-expressing HEK293 cells. n=3–5.

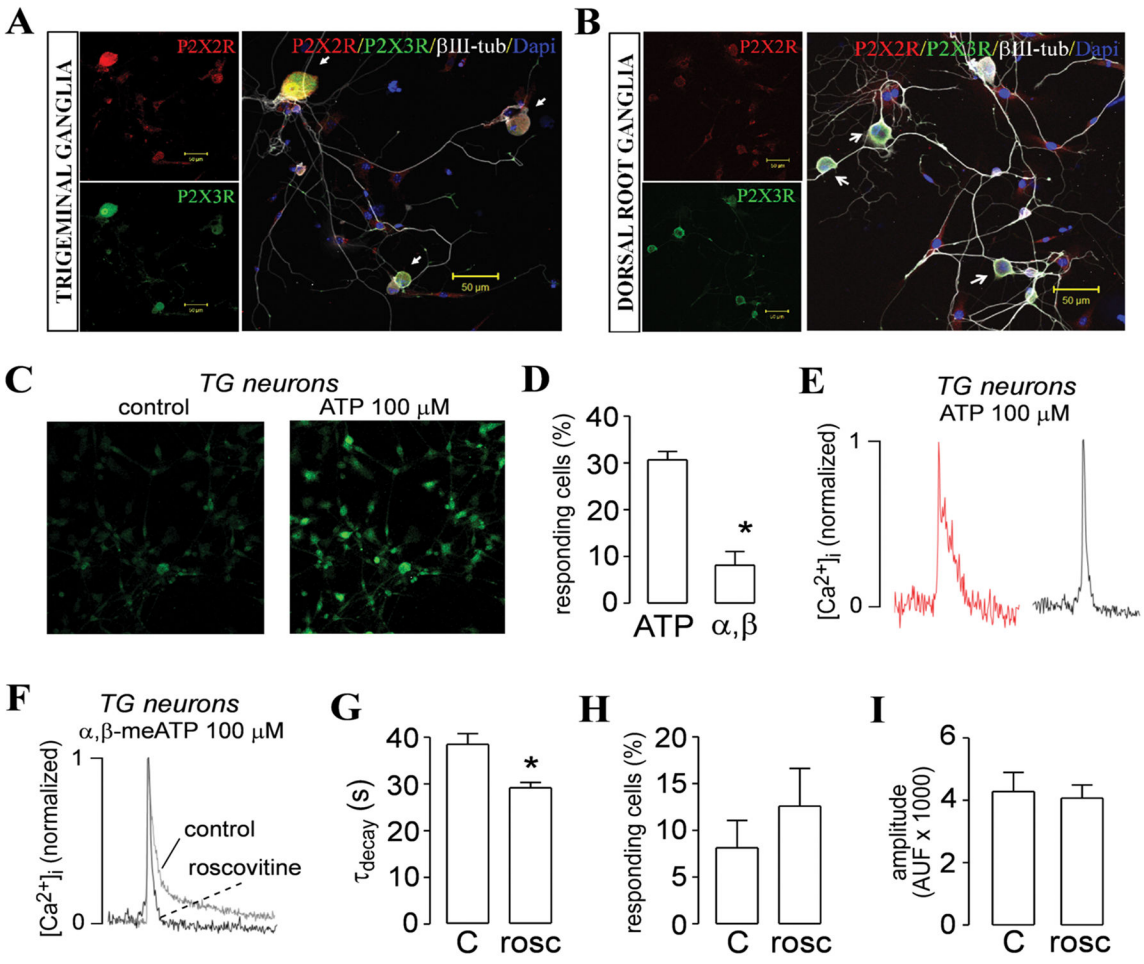

Figure 9. Cdk5 regulation of the heteromeric P2X2/3Rs

Confocal immunofluorescence of primary cultured neurons of 2 DIV from mouse TG (**A**) and DRG (**B**), expressing endogenous levels of P2X2R (red) and P2X3R (green). Neurons were immunostained with βIII-tubulin (white) and Dapi was used as nuclear staining. Arrows indicates neurons expressing heteromeric P2X2/3R subtype. **C.** Representative fields of cultured TG neurons loaded with Fluo-4 before (left image) and after (right image) the addition of 100 μM ATP. **D.** Summary of the percentage of responding cells to ATP and α,β-meATP (α,β). n=3, *p<0.05, student's t test. **E.** Typical Ca^{2+} spikes induced by 100 μM ATP. **F.** Representative Ca^{2+} spikes induced by 100 μM α,β-meATP without (control) or with a 6-hour roscovitine treatment (rosc). **G–I.** Summary of the results obtained in TG neurons without (C) or with the roscovitine treatment (rosc) for decay constant (**G**), percentage of responding cells (**H**) and relative amplitude (**I**). n=20–71; *p<0.05, Mann-Whitney test.