Mechanisms Mediating the Enhanced Gene Transcription of P2X$_3$ Receptor by Calcitonin Gene-related Peptide in Trigeminal Sensory Neurons*[S]

Manuela Simonetti[‡]**, Rashid Giniatullin**[‡§]**, and Elsa Fabbretti**[‡1]

From the ‡*Neurobiology Sector and Italian Institute of Technology Unit, International School for Advanced Studies (SISSA),*
34014 Trieste, Italy and §*A. I. Virtanen Institute, University of Kuopio, Neulaniementie 2, FIN-70211 Kuopio, Finland*

The molecular mechanisms underlying migraine pain remain unclear and probably require sustained facilitation in pain-sensing P2X$_3$ receptors gated by extracellular ATP in nociceptive sensory neurons. The major migraine mediator calcitonin gene-related peptide (CGRP) is known to sensitize P2X$_3$ receptors to increase impulse flow to brainstem trigeminal nuclei. This process is mediated via changes in the expression and function of P2X$_3$ receptors initially through enhanced trafficking and, later, perhaps through augmented synthesis of P2X$_3$ receptors. To clarify the mechanisms responsible for CGRP-evoked long lasting alterations in P2X$_3$ receptors, we used as a model mouse trigeminal ganglion neurons in culture. CGRP activated Ca^{2+}-calmodulin-dependent kinase II, which became localized to the perimembrane region and neuronal processes, a phenomenon already apparent after 30 min and accompanied by a parallel increase in cAMP-response element-binding protein (CREB) phosphorylation and nuclear translocation. These effects triggered increased P2X$_3$ receptor transcription and were prevented by expressing a dominant negative form of CREB. Increased P2X$_3$ receptor synthesis was partly mediated by endogenous brain-derived neurotrophic factor (BDNF) because of its block by anti-BDNF antibodies and mimicry by exogenous BDNF. Immunocytochemistry experiments indicated distinct subpopulations of BDNF- or CGRP-sensitive trigeminal neurons with only partial overlap. The present data indicate a novel mechanism for enhancing P2X$_3$ receptor expression and function in trigeminal sensory neurons by CGRP via CREB phosphorylation. BDNF was an intermediate to extend the sensitizing effect of CGRP also to CGRP-insensitive neurons. This combinatorial action could serve as a powerful process to amplify and prolong pain mediated by P2X$_3$ receptors.

Sensitization of sensory neurons represents the key phenomenon for generating chronic pain that remains relatively refractory to standard treatment (1). Current theories predict that chronic pain is brought about by a gain in the expression and function of sensory nociceptors that enhance their signal flow to the brain to ensure the long lasting nature of this process (2). Clarification of the novel gene expression profiles associated to pain should, therefore, be useful to target emerging molecules (and mechanisms) and for designing new analgesics. Previous studies (3) aimed at detecting changes in transcriptome patterns during pain conditions have provided only a limited description of the global alterations because of the complexity of pain pathologies and signal pathways.

Migraine is a classical example of chronic, relapsing pain triggered by local release of endogenous pain mediators, such as the neuropeptide calcitonin gene-related peptide (CGRP)[2] (4, 5) that induces long lasting trigeminal neuronal sensitization (6) and novel gene transcription in sensory neurons (7–9). CGRP-mediated signals are transduced at the cell membrane by activation of a G-protein-coupled receptor complex (composed by calcitonin receptor-like receptor, RAMP1 (receptor activity-modifying protein-1), and receptor component protein) that activates cAMP-dependent and Ca^{2+}-dependent signaling pathways (10, 11). Cultured trigeminal neurons are a useful model for investigating the mechanisms of migraine pain. On such sensory neurons, CGRP persistently and selectively up-regulates the membrane expression and activity of P2X$_3$ receptors (7) that are gated by extracellular ATP to transmit nociceptive stimuli to brainstem trigeminal nuclei (12, 13). This effect leads to long term sensitization of P2X$_3$ receptor function through increased receptor trafficking and neosynthesis (7). It is, however, unclear what intracellular mechanisms regulate the delivery and new supply of receptors to the plasma membrane and their time course. This is an important issue for the use of CGRP antagonists to treat migraine pain (14), because the administration time of these agents should take into account the dynamics of the intracellular events triggered by the peptide and their dependence on CGRP receptor activation. Additionally, it is clear that effective anti-migraine treatment should be initiated at the beginning of an attack (14), suggesting the need to clarify the earliest changes in nociceptive mechanisms. Because CGRP can elicit kinase-dependent gene transcription

[2] The abbreviations used are: CGRP, calcitonin gene-related peptide; CRE, Ca^{2+}/cAMP response element; CaMK, Ca^{2+}-calmodulin dependent kinase; CREB, cAMP-response element-binding protein; ChIP, chromatin immunoprecipitation; EGFP, enhanced green fluorescent protein; TG, trigeminal ganglion; GFP, green fluorescent protein; BDNF, brain-derived neurotrophic factor.

in a variety of cell systems (4), if a similar process occurs with $P2X_3$ receptors in trigeminal neurons, it might contribute to persistent pain. The present study sought to identify the early molecular mechanisms linking CGRP receptor activation to *P2X₃* gene transcription in cultured trigeminal neurons; the present data suggest that CGRP activated calcium/calmodulin-dependent kinase II (CaMKII) and the transcription factor cAMP-response element-binding protein (CREB) at least in part via release of the neurotrophin BDNF.

EXPERIMENTAL PROCEDURES

Animals and Cell Cultures—Primary cultures of C57Black/c mouse trigeminal ganglion (TG) sensory neurons were prepared and maintained in culture as previously described (16). For transfection experiments, neurons were transfected with the calcium/phosphate method (17), using 3 μg of EGFP-ACREB-encoding plasmid (provided by Dr. C. Giachino, University of Turin) (18, 19) or EGFP plasmid (Clontech and Takara Bio Inc.) and analyzed 48 h later. The following substances (all from Sigma) were added to the culture medium as required: CGRP (1 μM), CGRP-(8–37) (2 μM), KN-93 (3 μM), its inactive analog KN-92 (5 μM), AIP (autocamtide 2-related inhibitor peptide myristoylated) (1 μM), chelerythrine chloride (5 μM), H-89 (1 μM), forskolin (10 μM), mouse anti-BDNF neutralizing antibody (1 μg/ml), and BDNF (10 ng/ml) (20). The ability by the anti-BDNF antibody to block the action of BDNF was validated with Western blot experiments, in which BDNF-mediated CREB phosphorylation was fully prevented by anti-BDNF antibodies (supplemental Fig. 2). Furthermore, the same antibody blocked the increased $P2X_3$ mRNA evoked by BDNF in real time RT-PCR experiments (run in duplicate). The CGRP peptide antagonist or kinase inhibitors were preapplied for 30 min and then co-applied with CGRP for the indicated time.

Experimental protocols involving animals were approved by the local ethical committee and local authority veterinary service and run in accordance with the Italian Animal Welfare Act. For *in vivo* experiments, C57Black/c mice (6 animals, post-natal day 20) were subcutaneously injected (30 μl) in the neck with 1 μM CGRP. Five h later, animals were sacrificed, and their TG was explanted. This protocol was based on the reported clearance of CGRP calculated as 63.9 $\pm$ 4.5 min (21).

Real Time Reverse Transcription-PCR—Total mRNA extraction from TG cultures or ganglia and reverse transcription reactions was performed as previously reported (22), using the specific primers listed in Table 1. Data were normalized with respect to *GAPDH* and β-tubulin III housekeeping mRNA contents.

Western Immunoblot—TG cultures were processed for Western immunoblotting as described (16). The following polyclonal antibodies were used: anti-$P2X_3$ (1:300; Alomone, Jerusalem, Israel), β-tubulin III (1:3000; Sigma), anti-α CaMKII (1:500; Invitrogen), anti-phosphorylated Ser^{133} CREB (1:1000; Upstate and Millipore), anti-phospho-Thr^{286} CaMKII (1:1000; Promega (Madison, WI) and Upstate), and anti-GFP antibody (1:1000; made in house). Western signals were detected with the ECL system (Amersham Biosciences). Western blot signals were quantified measuring band density using Cyon Image and CorelDraw Photopaint software (Corel, Ottawa, Canada). Values of phosphorylated CaMKII or CREB signals were normalized *versus* the level of total CaMKII or β-tubulin III, used as loading control.

BDNF Release Assays—A dot blot immunoassay was used to detect the BDNF release in the medium of TG culture in control condition or after treatment with CGRP (1 μM, 30 min). Briefly, 50 μl of culture medium were spotted on a nitrocellulose membrane and incubated overnight with anti-BDNF antibody (1:250; Sigma). Signals were detected with anti-mouse horseradish peroxidase-conjugated antibody (1:1000; Sigma) and ECL.

A quantitative measure of BDNF concentration in the bulk medium was obtained with the Emax BDNF immunoassay system (Promega). TG culture medium was collected 30 min after CGRP application and immediately processed for the ELISA assay. Results were corrected for blank and normalized with respect to the genomic DNA content. The nonpeptide CGRP receptor antagonist BIBN4096BS (100 ng/ml; gift from Roche Applied Science) was also used for certain experiments.

Immunofluorescence Experiments—Immunofluorescence experiments (16) were performed with the following primary antibodies: anti-$P2X_3$ (1:200; Alomone), anti-phosphorylated Thr^{286} CaMKII (α type; 1:500; Promega), anti-phosphorylated Ser^{133} CREB (1:300; Upstate), β-tubulin III (1:1000, Sigma), anti-TrkB (1:200, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and the secondary antibody AlexaFluor488 or AlexaFluor594 (1:500; Invitrogen). To visualize neurons sensitive to CGRP, CGRP binding experiments were performed on live cells with rhodamine-conjugated CGRP (1 h at 4 °C; Phoenix-peptide, Belmont, CA) (23), as previously described (7). Rhodamine-conjugated-CGRP-labeled neurons were then processed for immunofluorescence using the anti-TrkB receptor antibody. Control CaMKII immunofluorescence experiments performed on CGRP-treated neurons treated with CaMK inhibitors or incubated for 1 h in Ca^{2+}-free medium (24) produced no signal (supplemental Fig. 1, *A* and *C*). For each experiment, an average of 500 cells were analyzed; data are the mean of at least three independent experiments. Results were quantified with MetaMorph software (Molecular Devices Corp., Downingtown, PA).

Chromatin Immunoprecipitation—For chromatin immunoprecipitation (ChIP) experiments, cultures obtained from six pairs of TGs were used for each condition in each experiment. Initial quantification of total genomic extracts was done with real time amplification analysis of genomic *GAPDH* content. ChIP experiments were performed using the ChIP assay kit (Upstate) following the manufacturer's instructions. Briefly, TG neurons, cross-linked with paraformaldehyde (1% in physiologic solution, 10 min, 37 °C), were sonicated (three pulses of 30 s each) to generate small genomic DNA fragments (<500 bp in length). Immunoprecipitation of cross-linked chromatin/protein extracts was performed using anti-phosphorylated Ser^{133} CREB antibody (active form, 5 μg; Upstate) overnight at 4 °C. The immunopurified genomic DNA fragments were amplified with real time PCR using specific primers (Table 1) spanning the Ca^{2+}/cAMP-response element (CRE) region of the *p2rx3* promoter (ENSMUSG00000027071) (25). As a positive control, cells treated with forskolin (10 μM, 10 min) and

immunopurified with anti-phospho-CREB antibody showed an significant binding to the NK1 tachykinin-1 receptor promoter region (*n* = 3; *tacr1*; ENSMUSG00000030043) detected with real time PCR and using specific primers (9). No PCR signal was obtained when genomic fragments where immunoprecipitated with unrelated anti-Egr1 antibody (*n* = 2; anti-Zif268; Santa Cruz Biotechnology). As ChIP negative controls, we amplified anti-CREB immunopurified fragments with additional primers (Table 1) spanning two different regions of the *p2rx3* promoter that lack the putative CRE sequence. Note that genomic region 1 contains a specific Erg-1 consensus sequence that was used as internal control. End point PCR amplicons were analyzed on agarose gel with ethidium bromide staining. Genomic analysis of *p2rx3* gene promoter regions was performed using the TFSEARCH data base (available on the World Wide Web) or the CREB Target Gene Data base (available on the World Wide Web).

Patch Clamp Recording—TG neurons were continuously superfused with physiological solution as previously reported (7). Cells were voltage-clamped (whole cell configuration) at −60 mV (70% series resistance compensation). The highly selective P2X₃ receptor agonist α,β-methylene ATP (10 μM, 2 s) was applied with a fast superfusion system (Rapid Solution Changer RSC-200; BioLogic Science Instruments). Inward current responses were measured in terms of peak amplitude. Electrophysiological data were always compared with responses from control sister dishes maintained *in vitro* for an equivalent time.

Statistical Analysis—Data are presented as the means ± S.E. of the mean (*n* = number of experiments). The statistical significance was assessed with the Mann-Whitney rank sum test and the Wilcoxon test for nonparametric data and with Student's *t* test for parametric data (KyPlot, version 2.0; Qualest Co.). A *p* value of <0.05 was accepted as indicative of a statistically significant difference.

RESULTS

Active CaMKII Is Necessary to Up-regulate P2X₃ mRNA Expression Induced by CGRP—Although previous experiments have indicated the time course of the CGRP-mediated increase in P2X₃ receptor expression and function (7), the identification of the early biochemical mechanisms involved in this effect has remained elusive and is a necessary step to formulate novel approaches to modulate trigeminal pain sensation. For this purpose, the present study sought to identify the transduction processes linking CGRP receptor activation to *P2X₃* gene transcription in cultured trigeminal neurons. We started by examining what kinase activity might mediate the action of CGRP. Because the action of CGRP is often mediated by kinase activity (4), we performed real time reverse transcription-PCR experiments on extracts from cultured TG neurons treated with CGRP (1 μM, 1 h) alone or co-applied together with kinases inhibitors to explore the signaling elements involved in CGRP-evoked P2X₃ receptor up-regulation. The PKA blocker H-89 (1 μM) or the PKC blocker chelerythrine (5 μM) partially prevented the potentiating effect of CGRP on P2X₃ mRNA transcription (*n* = 4, *p* = 0.03; Fig. 1*A*). Stronger inhibition of the action of CGRP could not be observed even when both blockers
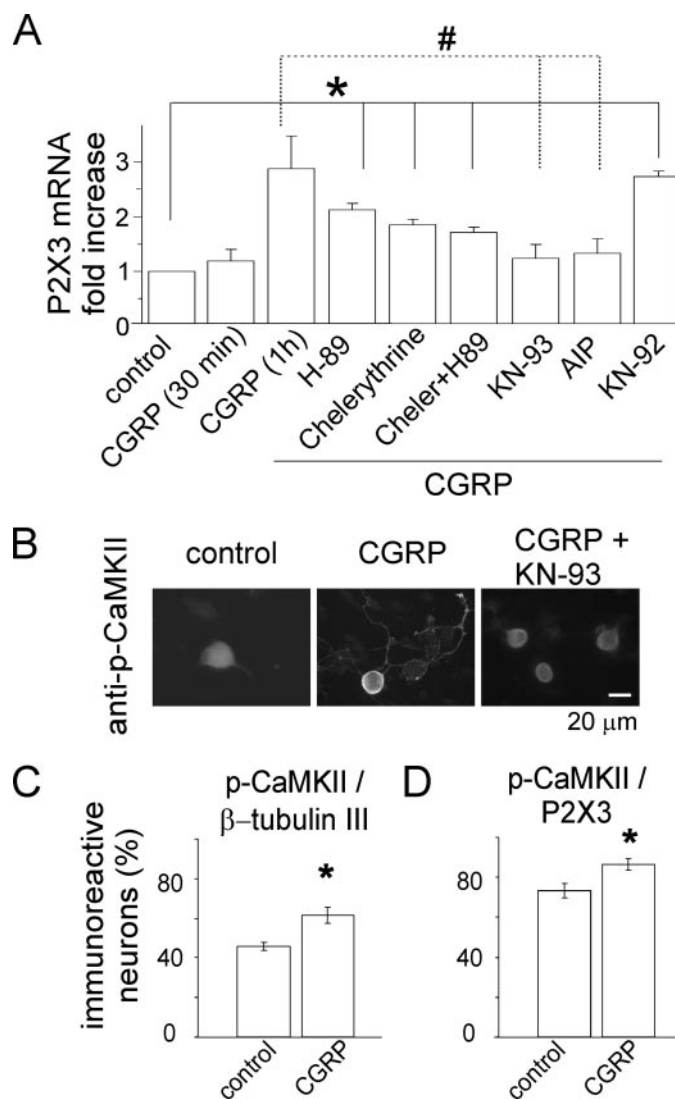

FIGURE 1. **CGRP regulates P2X₃ receptors via CaMKII.** *A*, real time reverse transcription-PCR experiments of TG neurons treated with CGRP and kinase inhibitors. CGRP (1 μM, 1 h) up-regulates P2X₃ mRNA neosynthesis, whereas co-application of CGRP with the CaMKII/IV inhibitor KN-93 (3 μM) or the CaMKII inhibitor AIP (1 μM) significantly blocks P2X₃ mRNA up-regulation (*n* = 3; #, *p* = 0.02), whereas the KN-92 congener is ineffective. Co-application of CGRP (1 μM, 1 h) with the PKA inhibitor (H-89, 1 μM) or the PKC inhibitor (chelerythrine, 5 μM) does not block the effect of CGRP on P2X₃ mRNA transcription. Co-application of H-89 and chelerythrine blocks only partially the effect of CGRP (*n* = 4; *, *p* = 0.03 with respect to control). *B*, microphotographs of TG neurons stained with the antibody against active phosphorylated Thr²⁸⁶ CaMKII (*p-CaMKII*). CaMKII activation in CGRP-treated neurons (*middle*) is completely prevented by KN-93 (3 μM, 1 h; *right*). *C* and *D*, the *histograms* show that the percentage of TG neurons with phosphorylated CaMKII in control and after CGRP application (1 μM, 1 h) taking as 100% either β-tubulin III (*C*) or P2X₃ (*D*) immunoreactive cells (*n* = 3 and *n* = 5 experiments; *, *p* = 0.04).

were co-applied (Fig. 1*A*). The specific inhibitor for CaMKII, AIP (1 μM, 1 h), or the CaMKII/IV inhibitor KN-93 (3 μM, 1 h) fully prevented CGRP-induced P2X₃ mRNA up-regulation (*n* = 3; #, *p* = 0.02; Fig. 1*A*). Conversely, application of KN-92 (5 μM, 1 h), an inactive congener of KN-93, was ineffective (*n* = 3, *p* > 0.05; Fig. 1*A*). Globally, the present results suggest a major role of CaMKII in CGRP-dependent P2X₃ mRNA neosynthesis. This notion was confirmed with immunocytochemical experiments using a phosphospecific antibody that recognizes only
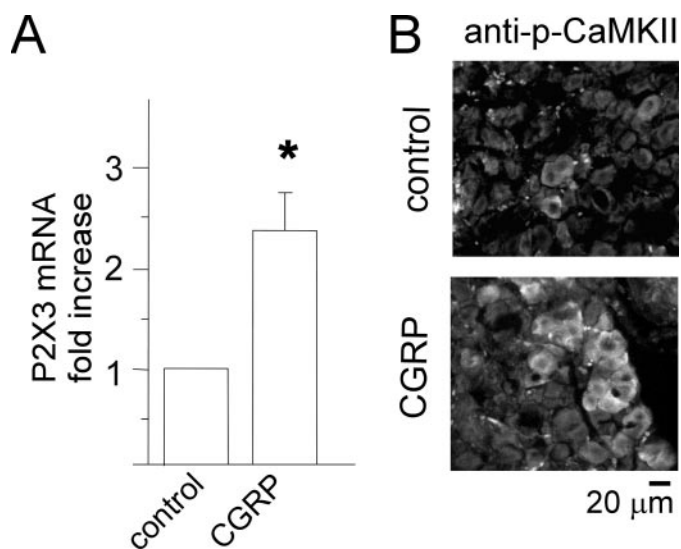
FIGURE 2. **Effects of *in vivo* administration of CGRP on P2X$_3$ mRNA and CaMKII.** *A*, real time PCR experiments indicate up-regulation of P2X$_3$ mRNA transcription in TG ganglion extracts from animals treated with CGRP (1 μM, 5 h; $n = 3$; *, $p = 0.04$). *B*, example of immunohistochemistry of TG ganglia from controls and CGRP-injected animals, using antibodies recognizing the active form of CaMKII. *Bar*, 20 μm.

FIGURE 3. **Time course of CaMKII activation in TG neurons.** *A*, example of immunofluorescence microphotographs of cultured TG neurons stained with anti-CaMKII antibody (recognizing the activated form only) at different time points from CGRP application (1 μM). *Pseudocolors* quantify the different levels of phosphorylated CaMKII in TG neurons under different conditions. Note that the localization of active CaMKII to the perimembrane region is completely prevented by co-application with KN-93. *B*, the *histograms* show the quantification of activated CaMKII signal that increases significantly after 30 min ($n = 3$; *, $p = 0.03$), and it is prevented by KN-93. *C*, Western immunoblot experiments of total TG culture with anti-active CaMKII antibody (52 kDa; *top*) after various times of exposure to CGRP. Total levels of CaMKII (52 kDa; α subunit) and neuronal β-tubulin III (55 kDa) gel loading quantification is also shown. The *histograms* quantify anti-CaMKII antibody signals (*bottom*), showing a significant increase after 30 and 60 min from CGRP application (*lanes 4* and *5*; $n = 3$; *, $p = 0.02$ and $p = 0.04$, respectively), prevented by co-application of KN-93 (1 h, 3 μM; *lane 6*, $n = 3$; $p > 0.05$).

the active form of CaMKII (anti-phospho-Thr[286] CaMKII antibody; see also supplemental Fig. 1). The example of Fig. 1*B* shows that, on a TG neuron, treatment with CGRP (1 μM, 1 h) not only led to stronger phosphorylated CaMKII kinase immunoreactivity but also to a different topographical distribution of this enzyme that acquired a mainly perimembrane location (Fig. 1*B*, *middle*). This effect was fully prevented by KN-93 (3 μM, 1 h; Fig. 1*B*, *right*) or AIP (1 μM, 1 h; not shown). Furthermore, although in control conditions the phosphorylated form of CaMKII was present in about 50% neurons (*i.e. β-tubulin III*-immunoreactive cells; $n = 5$ experiments; Fig. 1*C*) predominantly of small/medium somatic size (<25 μm), after CGRP treatment, their number rose to more than two-thirds (Fig. 1*C*). A significant increase in co-localization of P2X$_3$ and phosphorylated CaMKII immunoreactivity was also found ($n = 5$ experiments; Fig. 1*D*); this result shows that not only the distribution of the phosphorylated form of CaMKII (and number of neurons positive to it) was changed but that it was more often co-localized with the ATP-gated P2X$_3$ receptors. This observation is interesting, because, despite the limited number (35%) of trigeminal neurons responsive to CGRP *in vitro* (7), it outlines a potential mechanism for obtaining stronger nociception. Furthermore, the cell expression of the phosphorylated form of CaMKII was also enhanced (Fig. 3*C*).

In vivo experiments are necessary to validate data obtained with cell cultures; we, therefore, tested if CGRP could promote P2X$_3$ mRNA transcription also in mouse trigeminal ganglia *in situ*. To this aim, after injecting mice with saline or CGRP (subcutaneously into the neck, 1 μM, 10 μl), TG ganglia were explanted 5 h later and processed for real time PCR analysis or immunohistochemistry (Fig. 2). *In vivo* CGRP administration induced a significant up-regulation of P2X$_3$ mRNA neosynthesis in TG ganglia ($n = 3$, $p = 0.04$; Fig. 2*A*) and increased immunoreactivity for active CaMKII ($n = 3$, Fig. 2*B*). Globally, these data indicated that CGRP induced phosphorylation and redis-
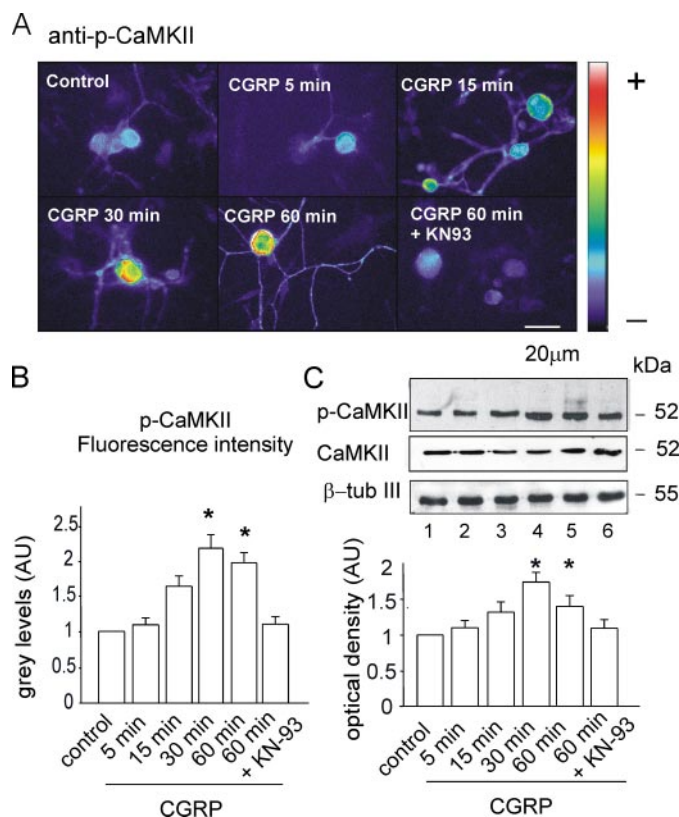
tribution of CaMKII necessary for *P2X$_3$* gene transcription in *in vivo* as well as in culture conditions.

Time Course of CGRP-dependent Activation of CaMKII—Short application of CGRP (1 μM; <30 min) was not sufficient to induce a significant up-regulation of P2X$_3$ mRNA transcription (1.3 ± 0.1-fold increase, $n = 3$, $p > 0.05$; Fig. 1*A*) by TG neurons in culture. To better resolve the time scale of CaMKII activation in the presence of CGRP, TG neurons were incubated with CGRP (1 μM) for 5, 15, 30, or 60 min and then processed for immunofluorescence or Western immunoblotting. Quantitative immunoreactivity analysis demonstrated that active CaMKII showed a stronger signal (see *red* and *white pseudocolors* in Fig. 3*A*) already after 15 min from CGRP application (Fig. 3, *A* and *B*), becoming significantly larger after 30 min ($n = 3$, $p = 0.03$), and fully prevented by KN-93 (Fig. 3, *A* and *B*). Furthermore, phosphorylated CaMKII immunoreactivity displayed predominant perimembrane localization and distribution throughout the neuronal processes (Fig. 3*A*, 60 min).

Western immunoblot experiments performed with total protein extracts of trigeminal ganglion cultures at different times from CGRP application confirmed the time-dependent increase of the band corresponding to active CaMKII (52 kDa; Fig. 3*C, top*). Even with total cell lysates, the phospho-CaMKII expression was significantly up-regulated already after 30 min of CGRP application ($n = 3$, $p = 0.02$; *lane 4*) and was maintained up to 1 h later ($n = 3$, $p = 0.04$; Fig. 3*C, lane 5*). This effect was not accompanied by a concomitant change in total CaMKII levels (52 kDa; Fig. 3*C* and supplemental Fig. 1). The increased CaMKII activation was not detected when KN-93 (3 μM, 1 h) or AIP (1 μM, 1 h) was co-applied with CGRP ($n = 3$, $p > 0.05$; Fig. 3*C* and supplemental Fig. 1, *A* and *B*), whereas KN-92 was ineffective (supplemental Fig. 1). Despite the low number of neurons responsive to CGRP and the presence of nonneuronal cells, the CaMKII response after CGRP treatment was fully prevented by co-application of CGRP with the CGRP receptor antagonist CGRP-(8 –37) (1.06 $\pm$ 0.1-fold increase, $n = 3$, $p > 0.05$; not shown).

CGRP Activates CREB in TG Neurons—Because of the lack of data about the transcriptional mechanisms involved in *P2X₃* gene expression, targeted experiments are required to identify novel regulatory elements controlling pain receptor expression in sensory neurons. Thus, we investigated whether *P2X₃* gene expression changes induced by CGRP might be mediated by the transcription factor CREB (8, 9), whose activation and nuclear location are strictly dependent on phosphorylation at Ser[133] by CaMKII (26). To investigate if CGRP-dependent CaMKII phosphorylation might activate CREB in TG neurons, cultures were analyzed at different times from CGRP application (1 μM; 5, 15, 30, 45, and 60 min; Fig. 4) with immunofluorescence and Western blot experiments using an antibody specific for the active form of CREB (anti-phospho-Ser[133] CREB). Although in control conditions only few neurons (β-tubulin III immunoreactive cells) were immunopositive for the phosphorylated form of CREB (Fig. 4*A*), the number of neurons displaying nuclear localization of active CREB increased in a time-dependent manner during CGRP treatment, reaching a significant level at 30 min ($n = 5$, $p = 0.02$; Fig. 4, *A* and *B*).

Accordingly, Western immunoblotting experiments demonstrated that the intensity of the band corresponding to phosphorylated CREB (α subtype; 43 kDa) was enhanced in a time-dependent manner, reaching a significant level 30 min after CGRP application ($n = 3$, $p = 0.04$; Fig. 4*C, lane 4*) and growing up to 1 h ($n = 3$, $p = 0.025$; Fig. 4*C, lane 5*). The activation of CREB and its nuclear localization were completely prevented ($n = 3$) when CGRP (1 μM, 1 h) was co-applied with KN-93 (3 μM, 1 h) (Fig. 4, *A*, *B*, and *C, lane 6*). Since CREB phosphorylation in P2X₃-positive neurons was fully blocked by the CaMKII inhibitor, these data suggest that, notwithstanding the ability of multiple pathways to phosphorylate CREB Ser[133], this site was an essential step in the action of CGRP on sensory neurons.

CREB Binds the p2rx3 Gene Promoter to Activate P2x₃ mRNA Transcription—In order to activate gene transcription, CREB interacts with genomic regulatory elements possessing the specific consensus signal. Thus, to demonstrate the involvement of CREB in *P2X₃* gene transcription of trigeminal neurons, we performed ChIP assays using the anti-phospho-CREB
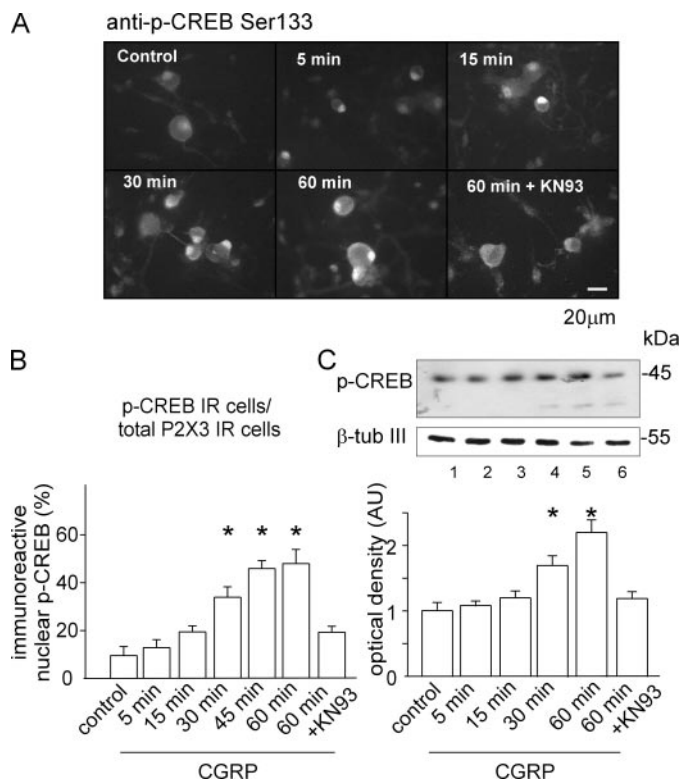

FIGURE 4. **CGRP activates CREB in TG neurons.** *A*, example of immunofluorescence microphotographs of TG neurons stained with antibody against the active form of CREB (anti-phospho-Ser[133]) at different times from initial CGRP (1 μM) application. Note that the localization of CREB to the nucleus is prevented by KN-93. *B*, the *histograms* show the percentage of P2X₃-immunoreactive neurons with nuclear CREB signal; their value is significantly increased 30 min after initial CGRP application ($n = 5$; *, $p = 0.03$). *C*, Western immunoblot experiments of neurons at different times from CGRP application (*top*) using the anti-phospho-CREB antibody (43 kDa). Gel loading quantification is obtained using anti-β-tubulin III antibody. Analysis of anti-CREB Western blot shows significant CREB phosphorylation already at 30 min from CGRP application (*lane 4*; $n = 3$; *, $p = 0.04$) and further rising at 1 h (*lane 5*, $n = 3$; *, $p = 0.025$). This effect is prevented by KN-93 (3 μM, 1 h; *lane 6*; $n = 3$, $p > 0.05$).

antibody to immunopurify the CREB-associated *p2rx3* gene promoter in control conditions and after CGRP application (1 μM, 1 h). Fig. 5*A* shows an example of how CGRP induced CREB binding to the *p2rx3* promoter under conditions of equivalent loading estimated with the amplification of the housekeeping *GAPDH* gene. These observations were quantified with real time PCR that confirmed that CGRP significantly increased the immunopurification of *p2rx3* genomic CRE sequence together with phospho-CREB ($n = 3$, $p = 0.02$; Fig. 5*B*). As ChIP negative controls, we amplified, with real time PCR (before and after CGRP treatment), anti-CREB immunopurified fragments with additional primers (Table 1) spanning two different regions of the *p2rx3* promoter that lack the CRE sequence (see "Experimental Procedures"). These experiments gave no signal (see Fig. 5*B, left*, for the amplicons of genomic region 1). Similar data were obtained with the genomic region 2 (not shown). To confirm the method reliability, we ran the ChIP assay for the transcription factor Egr-1, which was successfully amplified (with the primers for genomic region 1) and found to be insensitive to CGRP treatment (Fig. 5*B, right*).

Nuclear localization of active CREB and its binding to specific promoter regions might be insufficient to activate specific
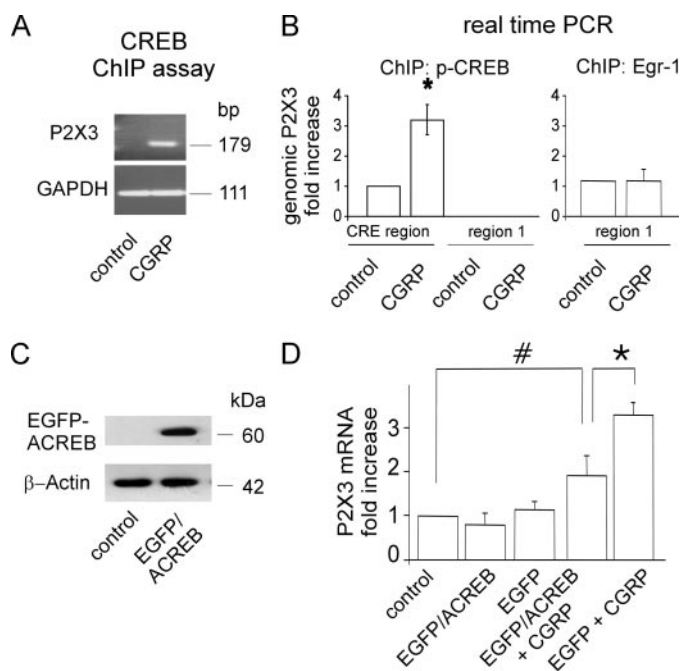
FIGURE 5. **ChIP assays of CGRP-treated TG neurons.** *A*, an agarose gel photograph shows an example of end point amplification of *p2rx3* CRE genomic region extracted from TG neurons in control conditions and after CGRP application (1 μM, 1 h) and purified with a ChIP assay using anti-phospho-Ser[133] CREB antibodies. Equal amounts of total input DNA were checked with *GAPDH* amplification. *B*, the *histogram* shows quantitative real time PCR analysis for *p2rx3* CRE genomic region purified with ChIP. CGRP significantly enhances immunopurification of *p2rx3* promoter CRE sequence together with CREB using genomic primers spanning CRE region (*left*; $n = 3$; *, $p = 0.02$). Negative controls of the ChIP assay were performed using genomic primers spanning regions that do not contain the CRE sequence (region 1) of the *p2rx3* gene with real time PCR before and after CGRP treatment and CREB immunopurification ($n = 3$). Note that, despite ChIP immunopurification of the genomic region 1 with anti-Egr1 antibody (*right*), no signal is detected with anti-phospho-CREB antibody (*middle*). *C*, example of Western immunoblotting of TG culture lysates transfected with EGFP-ACREB; the signal is obtained with the anti-GFP antibody and reflects the effective expression in TG culture of EGFP-ACREB fusion protein (~60 kDa). Gel loading quantification is obtained using anti-β-actin antibody (42 kDa). *D*, real time PCR experiments of mRNA extracts from TG neurons transfected with EGFP or dominant negative CREB (ACREB) in control conditions and after CGRP application (1 μM, 1 h). Neurons expressing EGFP-ACREB show a significantly reduced CGRP-induced P2X₃ mRNA synthesis ($n = 3$; #, $p = 0.03$; *, $p = 0.04$).

transcriptional programs or may outlast former transcription of CREB-dependent genes (27). For these reasons, we decided to perform additional experiments using a dominant negative form of CREB fused at the N terminus to EGFP (EGFP-ACREB) (19). Microscopy analysis of EGFP in TG cultures revealed a transfection efficiency of 15–20% (with respect to β-tubulin immunoreactive neurons); supplemental Fig. 3 shows an example of a TG neuron expressing recombinant EGFP-ACREB. The expression of the EGFP-ACREB fusion protein was further detected with Western immunoblotting using an anti-GFP antibody (Fig. 5C; apparent mobility ~60 kDa). Fig. 5D shows the result of real time PCR experiments performed on mRNA extracted from TG neurons treated with CGRP (1 μM, 1 h) after transfection with EGFP-ACREB plasmid. Expression of EGFP-ACREB or EGFP plasmids did not change the basal level of P2X₃ mRNA ($n = 3$, $p > 0.05$; Fig. 5D). However, in TG neurons, EGFP-ACREB expression inhibited *P2X₃* gene transcription induced by CGRP ($n = 3$; #, $p = 0.03$ with respect to untransfected cells; *, $p = 0.04$ with respect to EGFP expressing

plus CGRP; Fig. 5D). These data provided strong evidence for CGRP-mediated activation of CREB to induce P2X₃ mRNA transcription.

BDNF Promotes P2X₃ mRNA Up-regulation in TG Neurons— Because it was recently reported that CGRP can promote BDNF secretion by TG neurons (28) and BDNF is thought to be involved in pain processing (29), we investigated if BDNF could have a role in the CGRP-mediated sensitization of TG neurons via enhanced *P2X₃* gene transcription. Fig. 6A shows the distribution histogram of immunoreactive neurons for the BDNF receptor TrkB superimposed with neurons that bind CGRP. The majority (69 ± 3%) of neurons expressing TrkB had a mean somatic diameter of 15 μm, whereas the CGRP-binding neurons (7) usually had larger diameter (>20 μm). These results suggest the existence of two subpopulations of TG neurons sensitive to either CGRP or BDNF, with partial overlap (32 ± 4%) in the case of neurons with a 15–20-μm diameter range.

Dot blot analysis was performed to measure BDNF released in the bulk medium of TG cultures after 30 min of CGRP application. CGRP (1 μM) induced a 4-fold BDNF increase ($n = 7$, $p < 0.05$), not observed when the CGRP receptor antagonist BIBN4096BS (100 ng/ml) was co-applied together with CGRP ($n = 3$, $p > 0.05$). An enzyme-linked immunosorbent assay demonstrated that CGRP increased the BDNF concentration in the culture medium from 2.4 ± 0.2 to 8.2 ± 1.4 pg/ml ($n = 4$, $p < 0.05$); this effect was not found when BIBN4096BS was co-applied with CGRP (3.0 ± 0.3 pg/ml; $n = 3$, $p > 0.05$).

We next investigated the degree of BDNF involvement in mediating the action of CGRP on P2X₃ receptor synthesis. As shown in Fig. 6B, a 1-h co-application of CGRP (1 μM) and a mouse neutralizing anti-BDNF antibody (1 μg/ml) significantly attenuated the CGRP-induced P2X₃ mRNA up-regulation ($n = 3$; #, $p = 0.04$ with respect to control; *, $p = 0.03$ with respect to CGRP-treated), whereas application of anti-BDNF antibody alone did not show any significant change ($n = 3$, $p > 0.05$).

Patch clamp recording from TG neurons was used to investigate whether the anti-BDNF treatment could actually prevent the large facilitation of P2X₃ receptor-mediated inward currents evoked by 1-h treatment with CGRP (1 μM). Fig. 6C (*top traces*) shows that the increased peak amplitude of the current evoked by pulse application of α,β-methylene ATP (10 μM) after a 1-h exposure to CGRP was clearly smaller when the neuropeptide was co-applied for 1 h with the anti-BDNF antibody. On average, although the increment in peak current amplitude mediated by P2X₃ receptor activation was more than 2-fold after CGRP ($n = 34$ for control cells, and $n = 33$ for CGRP-treated cells; $p = 0.0001$), on neurons pretreated with CGRP plus the anti-BDNF antibody, the up-regulation was significantly less (~50%; $n = 13$ cells, $p = 0.02$ *versus* CGRP-treated). These data indicate that about half of the action of CGRP on P2X₃ receptors was mediated by BDNF; this observation raised the question of whether the latency in the effects of CGRP (7) was perhaps attributable to delayed release/action of BDNF. To examine this possibility, we compared the effect of a 30-min application of either CGRP (1 μM) or BDNF (10 ng/ml) on P2X₃ mRNA up-regulation (Fig. 6D). Although this neurotrophin did induce a significant increase in P2X₃ mRNA transcription ($n = 3$, $p = 0.03$; Fig. 6D), CGRP did not (Fig. 1A). We

TABLE 1

Experimental parameters used for PCR experiments

Listed are the primers used for real time PCR amplifications of genomic sequences or mRNA transcripts. The length of the amplified fragments (expressed in bp) and the temperature (T) of primer annealing are also indicated.

Name	Primers	Length	T
		bp	*°C*
Primers for genomic sequences			
CRE-containing genomic *p2rx3*			
Forward	5′-GCAGATGAAACATAAGTCC-3′	179	55
Reverse	5′-TCTCTCCTCTTGACACTC-3′	179	55
Genomic *GAPDH*			
Forward	5′-CACCCACTCCTCCACCTTTGAC-3′	111	55
Reverse	5′-TCCACCACCCTGTTGCTGTAG-3′	111	55
Egr-1-containing genomic region 1			
Forward	5′-AACCTCCACCAGCCCAAC-3′	123	55
Reverse	5′-TTCCACCAGAATGACGATGC-3′	123	55
Egr-1-containing genomic region 2			
Forward	5′-GATGTCGTTGAACTCCAATGTC-3′	184	55
Reverse	5′-TTCTTGCTTATCAGTCTCCTTCC-3′	184	55
Primers for mRNA			
P2X₃			
Forward	5′-GTCCTCACACATCCACACATCTG-3′	184	56
Reverse	5′-ACTCTGCCTACTCAACTACATCCC-3′	184	56
β-Tubulin III			
Forward	5′-CGCCTTTGGACACCTATTC-3′	240	56
Reverse	5′-TACTCCTCACGCACCTTG-3′	240	56
GAPDH			
Forward	5′-AGAAGGTGGTGAAGCAGGCATC-3′	123	56
Reverse	5′-CGAAGGTGGAAGAGTGGGAGTTG-3′	123	56

next explored if, in TG neurons, BDNF led to phosphorylation of CaMKII and CREB, thus stimulating downstream mechanisms analogous to those evoked by CGRP. Western immuno-blotting experiments (Fig. 6*E* and supplemental Fig. 1*C*) showed that a 30-min application of BDNF (10 ng/ml) indeed promoted CaMKII and CREB activation in TG neurons (1.4 ± 0.1- and 1.9 ± 0.2-fold increase, respectively; $n = 3$, $p = 0.04$, and $p = 0.03$). Our results indicated that BDNF mediated a large component of the action of CGRP on P2X₃ receptors of TG neurons.

DISCUSSION

Understanding the changing profile of neuronal gene expression during experimental pain may help to identify key elements involved in neuronal sensitization underlying persistent pain (30). The present work focused on the intracellular mechanisms mediating the delayed effects of the algogenic neuropeptide CGRP on P2X₃ receptors of trigeminal neurons, because overactivity of such receptors is believed to be responsible for enhanced pain signaling (12, 13). Nevertheless, why and how these receptors can remain overactive and perpetuate pain is still essentially unknown. This is a subject that requires close investigation, because identifying regulatory transcriptional mechanisms might help to design new analgesic approaches to chronic trigeminal pain.

In a model of trigeminal neurons in culture, CGRP triggered a signaling cascade that led to CaMKII and CREB activation to stimulate novel *P2X₃* gene expression, a process at least in part mediated via endogenous BDNF release. The present data, thus, provide a novel contribution to clarifying how pain-inducing substances like CGRP and BDNF, usually present in the extracellular microenvironment during a pain attack, may enhance expression and function of purinergic P2X₃ nociceptors and support long lasting neuronal sensitization.

Molecular Elements Involved in P2X₃ Gene Transcription Evoked by CGRP—Activation of the trigeminal vascular system at meningeal level during migraine implies the action of the neuropeptide CGRP, which causes mast cell degranulation, NO synthesis, and vasodilation together with sustained increase in TG sensory neuron responsiveness (6, 31). Since increased plasma levels of CGRP are found in patients within 1 h of the start of the migraine attack (5, 32), blocking CGRP effects is considered therapeutically relevant for migraine patients (32–35). TG neurons innervating meningeal vessels indeed secrete CGRP (36) and express its receptor (10). In TG neurons in culture, CGRP induces a delayed, long lasting up-regulation of P2X₃ receptors (7). In the present study, this effect required activation of several kinases, of which CaMKII played an essential role, since its pharmacological block fully prevented the action of CGRP on P2X₃ receptor synthesis. CaMKII has a fundamental role in establishing long term memory in hippocampal cells and neuronal plasticity usually mediated by activation of CREB (37–39). CREB is a prototypical stimulus-inducible transcription factor that regulates the expression of genes important for adaptive neuronal responses (40) as well as complex functions, such as learning and memory (41). In neurons, target genes activated by CREB are immediate early genes (like c-*fos*), molecules important for synaptic function (like BDNF), and neuronal nitric oxide synthase (42), all involved in pain mechanisms (31, 43). The ability of CREB to activate gene transcription is strictly dependent on extracellular stimuli that trigger CREB phosphorylation at the Ser[133] residue via multiple kinases, such as PKA, CaMKII, and CaMKIV (26, 42). Because CGRP is known to increase intracellular free Ca^{2+} (44), it is likely that this is a mechanism to activate CaMKII-dependent CREB phosphorylation (27, 45) in a subset of trigeminal neurons responding to CGRP.
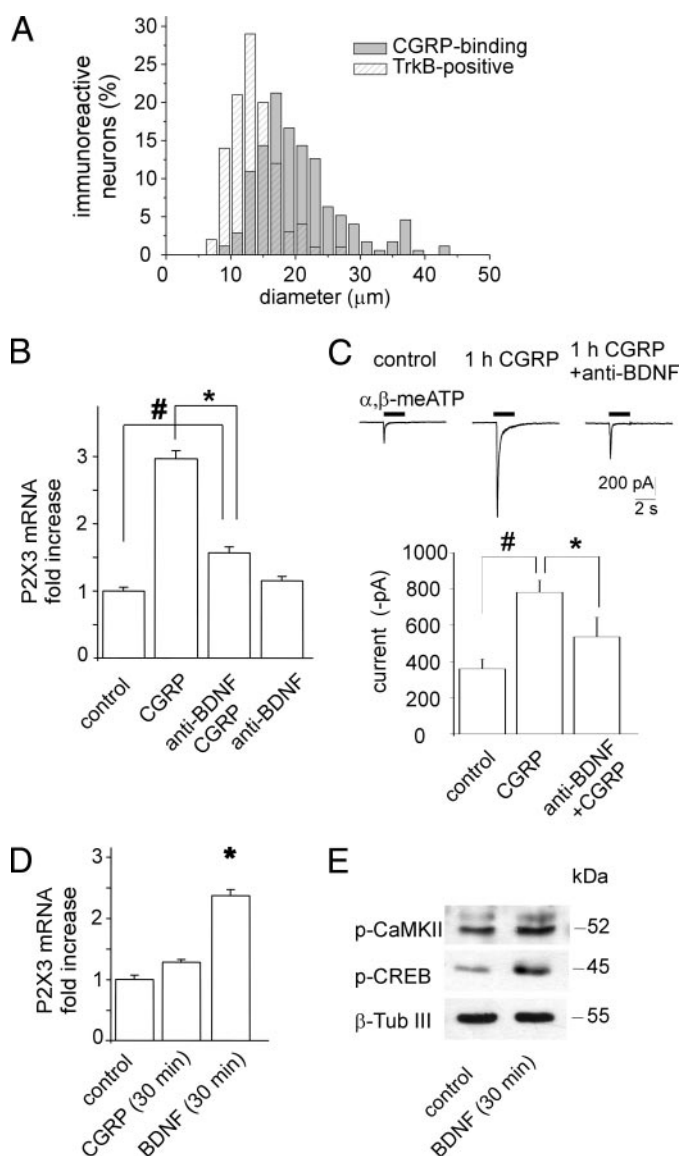
FIGURE 6. **BDNF effects on TG neurons.** *A*, somatic size distribution of TG cells immunostained with TrkB receptor antibody (*dashed bars*) or labeled with rhodamine-conjugated CGRP peptide (*filled bars*). Data are from 1000 cells ($n = 3$). *B*, real time PCR experiments of mRNA from TG neurons treated with CGRP (1 μM, 1 h) or with CGRP plus the anti-BDNF neutralizing antibody (1 μg/ml, 30 min). Anti-BDNF antibody partially reduces the effect of CGRP ($n = 4$; *, $p = 0.03$ *versus* CGRP-treated culture; #, $p = 0.04$ *versus* control culture). *C*, representative examples of currents induced by α,β-methylene ATP (*black bar*) on TG neurons cultured in control condition (*left*), after treatment with CGRP (1 μM, 1 h; middle), and with CGRP plus anti-BDNF antibody (1 μg/ml, 1 h; *right*). The *histograms* show that anti-BDNF partially blocks the effect of CGRP ($n = 13$ cells; *, $p = 0.03$ *versus* CGRP-treated culture; #, $p = 0.04$ *versus* control culture). *D*, real time PCR experiments of mRNA from TG neurons treated for 30 min with CGRP (1 μM) or with BDNF (10 ng/ml). Although application of BDNF (30 min) is able to induce P2X₃ mRNA ($n = 3$; *, $p = 0.03$), short application of CGRP does not have this effect. *E*, Western immunoblotting experiments of lysates from TG neurons in control conditions and after BDNF application (10 ng/ml, 30 min) analyzed with anti-active CaMKII and anti-active CREB antibodies ($n = 3$, $p = 0.03$). β-Tubulin III signal is used as gel loading control.

Although enhanced P2X₃ mRNA signals required at least a 1-h application of CGRP, this neuropeptide actually elicited earlier intracellular changes, because CaMKII activation and nuclear translocation of CREB occurred after just 30 min. These effects were sufficient to induce a specific genetic pro-

gram in TG neurons *in vivo* and *in vitro*, leading to novel transcription of pain receptor P2X₃. CREB activation was necessary for P2X₃ neosynthesis, since the competition of endogenous CREB with a dominant negative CREB mutant (19) blocked CGRP-induced P2X₃ mRNA synthesis. In fact, a CREB regulatory element (CRE-like sequence) is conserved in the rat, mouse, and human *P2X₃* gene promoter and is putatively responsible for binding active CREB (score 86.1%; TGACATCA sequence). Furthermore, in broad range genetic screening programs, the *P2X₃* promoter sequence was reported to be part of the "CREB regulon," a group of genes subjected to CREB regulation (46). We propose that CGRP-dependent up-regulation of P2X₃ mRNA may account for the long lasting potentiation of P2X₃ receptors (7), similar to what is reputed to occur at the meningeal level during a migraine attack (47). Likewise, CGRP stimulates CREB-dependent gene expression of other pain genes in primary afferents and spinal neurons (8, 9).

The present study arises from a previous investigation into gene expression (measured with microarray analysis) by meningeal tissue after an experimental migraine attack (48). That study showed complex variations in gene expression patterns, possibly due to the choice of the tissue samples that included a heterogeneous peripheral district. Our work elected to test trigeminal ganglion neurons as an advantageous model for receptor plasticity evoked by pain mediators.

Effect of BDNF—Neurotrophins, such as NGF and BDNF, play key roles in the development of inflammation-induced hyperalgesia (49, 50), triggering the neosynthesis of pronociceptive genes in primary afferents and spinal neurons, thus contributing to the development of persistent pain states (51). Nonetheless, NGF has minimal ability to activate CREB-mediated gene transcription (52–54) as well as *P2X₃* gene expression in TG neurons (22). In the present investigation, BDNF was an intermediate of the CGRP-induced events and was, *per se*, an adequate stimulus for *P2X₃* gene transcription in TG neurons as much as CGRP itself. Neutralization of endogenous BDNF largely prevented transcription of P2X₃ receptor mRNA by CGRP, presumably by inhibiting the common intracellular pathway of CaMKII and CREB. This finding is consistent with the report that the BDNF-encoding gene is a CREB target (55). The end result of blocking BDNF was a strong reduction in the ability of CGRP to generate larger electrophysiological responses mediated by P2X₃ receptors.

A Hypothesis for the Long Term Algogenic Action of CGRP on TG Neurons—We propose that cultured TG neurons could release enough endogenous BDNF for P2X₃ receptor up-regulation only when they were stimulated with CGRP, since the anti-BDNF antibody alone did not alter basal P2X₃ mRNA transcription.

Not all effects by CGRP were mediated by endogenous BDNF. This observation may be explained with the fact that there is partial segregation between subpopulations of neurons binding CGRP (usually of larger diameter) and expressing the BDNF receptor TrkB (typically of smaller diameter). Conversely, the majority of TG neurons express P2X₃ receptors (7, 16). Hence, comparatively large CGRP-binding neurons were

perhaps responsible for retaining part of the CGRP-evoked facilitation of P2X$_3$ receptor expression and function when BDNF was neutralized.

Our present findings raise some intriguing implications for designing novel approaches to block P2X$_3$ receptor sensitization, especially since BDNF levels are indeed increased in migraine patients during the acute phase of the pain attacks (56, 57). Pharmacological antagonism of CGRP receptors (33, 35) may be an effective analgesic protocol as long as BDNF is not released in a CGRP-independent fashion to perpetuate the P2X$_3$ receptor up-regulation. Nevertheless, during migraine, generation of NO (6, 58) can stimulate BDNF expression (59) and even control CREB-mediated gene expression in neurons (60).

Because multiple extracellular signals (CGRP, BDNF, NO) may converge on CREB activation, effective pain relief might, theoretically, need an approach targeted to selectively block CREB prior to its nuclear translocation. This strategy may aim at preventing the slowly developing onset of sensitization. Since ATP-sensitive receptors are potential targets for novel drugs against inflammatory pain (15, 61), the characterization of the mechanisms involved in P2X$_3$ neosynthesis and function may supply novel information helpful to design new therapeutics for neurophatic pain syndromes, including migraine, that remain poorly amenable to treatment in many patients.

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Mechanisms Mediating the Enhanced Gene Transcription of P2X$_3$ Receptor by Calcitonin Gene-related Peptide in Trigeminal Sensory Neurons

Manuela Simonetti, Rashid Giniatullin and Elsa Fabbretti

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