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Activation of cyclin-dependent kinase 5 broadens action potentials in human sensory neurons

Manindra Nath Tiwari[1,*], Bradford E Hall[2,*], Anh-Tuan Ton[3], Re Ghetti[3], Anita Terse[2], Niranjana Amin[4], Man-Kyo Chung[1], and Ashok B Kulkarni[2]

Abstract

Chronic pain is one of the most devastating and unpleasant conditions, associated with many pathological states. Tissue or nerve injuries induce extensive neurobiological plasticity in nociceptive neurons, which leads to chronic pain. Recent studies suggest that cyclin-dependent kinase 5 (CDK5) in primary afferents is a key neuronal kinase that modulates nociception through phosphorylation under pathological conditions. However, the impact of the CDK5 on nociceptor activity especially in human sensory neurons is not known. To determine the CDK5-mediated regulation of human dorsal root ganglia (hDRG) neuronal properties, we have performed the whole-cell patch clamp recordings in neurons dissociated from hDRG. CDK5 activation induced by overexpression of p35 depolarized the resting membrane potential (RMP) and reduced the rheobase currents as compared to the control neurons. CDK5 activation changed the shape of the action potential (AP) by increasing AP -rise time, -fall time, and -half width. The application of a prostaglandin E2 (PG) and bradykinin (BK) cocktail in control hDRG neurons induced the depolarization of RMP and the reduction of rheobase currents along with increased AP rise time. However, PG and BK applications failed to induce any significant changes in the p35-overexpressing group. We conclude that, in dissociated hDRGs neurons, CDK5 activation through the overexpression of p35 broadens the AP and that CDK5 may play important roles in the modulation of AP properties in human primary afferents under the condition in which CDK5 is upregulated, contributing to chronic pain.

Keywords

Action potential, cyclin-dependent kinase 5, human, inflammation, pain, sensory neurons

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[1]Department of Neural and Pain Sciences, School of Dentistry, Program in Neuroscience, Center to Advance Chronic Pain Research, The University of Maryland, Baltimore, MD, United States
[2]Functional Genomics Section, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD, United States
[3]AnaBios, San Diego, CA, United States
[4]National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD, USA

*These authors made equal contributions.

Corresponding Authors:
Man-Kyo Chung, Department of Neural and Pain Sciences, School of Dentistry, Program in Neuroscience, Center to Advance Chronic Pain Research, The University of Maryland, 650 W. Baltimore St., Baltimore, Maryland 21201, United States.
Email: MChung@umaryland.edu

Ashok B Kulkarni, Functional Genomics Section National Institute of Dental and Craniofacial Research National Institutes of Health, 30 Convent Drive, Room 130, Bethesda, MD 20892, United States.
Email: ashok.kulkarni@nih.gov

Introduction

Chronic pain affects about 20% of adults in the United States and for its effective treatment, the development of novel efficacious therapies without addiction liability are urgently required.[1] With ongoing pathological pain signaling, neuroplastic changes can occur that give rise to hyperalgesia and allodynia.[2] Peripheral sensitization, for instance, can arise with transcriptional and post-translational modifications downstream of inflammation. This generally includes the activation of protein kinases such as PKA, PKC, and Cyclin-dependent kinase 5 (Cdk5) in primary afferent neurons.[3] These protein kinases can, in turn, phosphorylate ion channels involved in pain signaling and then can lower their threshold of activation, affect channel desensitization, or enhance channel trafficking to the plasma membrane. Although Cdk5 shares a high degree of homology with other Cdk's, it is predominantly active in post-mitotic neurons, which makes it unusual from other Cdk's that are mostly involved in cell cycle regulation.[4] In addition, Cdk5 activity is neither induced by a cyclin, as typical with other Cdk family members, nor with a second messenger molecule as seen with PKA, PKC, and the Ca^{2+}/calmodulin-dependent protein kinase CaMKII. Instead, Cdk5 activity is regulated through the expression of its regulatory subunit p35, which binds with Cdk5 through a cyclin box. The upregulation of p35 occurs in the dorsal root ganglia following peripheral inflammation of the rodent hind paw via carrageenan[3] or complete Freund's adjuvant[5] or with neuropathic injury by spinal nerve ligation.[6] Further studies have shown that inflammatory cytokines including tumor necrosis factor α, transforming growth factor β, and nerve growth factor can trigger ERK1/2 activation to subsequently boost p35 expression and ultimately lead to increased Cdk5 activity.[4,7,8]

Cdk5 hyperactivity in primary afferent neurons has consequently been implicated with pain hypersensitivity. The transgenic overexpression of p35 alone can, in effect, promote both thermal and mechanical pain in mice.[3,9] This has been partially attributed to Cdk5 mediated phosphorylation of pain transducing ion channels such as the thermosensor transient receptor potential vanilloid subtype 1 (TRPV1),[10,11] the chemosensor transient receptor potential ankyrin subtype 1 (TRPA1),[12,13] and the purinergic receptor P2X2.[14] For example, phosphorylation by Cdk5 can potentiate pain signaling by curbing channel desensitization in TPRV1.[11] In addition, the dysregulated trafficking of voltage-gated ion channels has also been associated with chronic pain, where Cdk5 is known to phosphorylate both Cav3.2, causing enhanced trafficking to the plasma membrane, and CRMP2, which affects the trafficking of Nav1.7 and Cav2.2.[15,16]

Pathological pain conditions are accompanied by altered electrophysiological properties (action potential kinetics and intrinsic excitability).[17,18] The electrical properties of neurons including action potential spike width and spike threshold are tightly regulated by the ionic gradients, ion channel activation/deactivation time and their state of phosphorylation/dephosphorylation.[19,20] Despite the association of upregulated Cdk5 activity with hyperalgesic conditions, the role of Cdk5 in the modulation of human sensory neuronal properties has not been fully elucidated. Recently, electrophysiological recording of dissociated hDRG neurons has been possible through organ-donor networks.[21–24] Single nucleus sequencing shows that *CDK5* and its activator p35 (*CDK5R1*) are widely expressed in hDRG neurons.[25] So, an overall assessment of the effects of Cdk5 hyperactivity and other pain associated protein kinases on hDRG neurons has become feasible. In this study, to extend our previous preclinical studies on the roles of Cdk5/p35 in pain, we examined the effects of Cdk5 activation on hDRG neuronal electrophysiological properties. We aimed to determine the effects of exogenous p35 overexpression on the changes in the excitability, action potential properties, and responses to inflammatory mediators in dissociated human DRG neurons in culture.

Materials and methods

Human subjects

DRGs used in this study were recovered by AnaBios (San Diego, CA) from seven cadaveric donors with the informed consent of the next of kin. AnaBios Corporation's procurement network includes only US-based Organ Procurement Organizations and Hospitals. Policies for donor screening and consent are the ones established by the United Network for Organ Sharing (UNOS). Organizations supplying human tissues to AnaBios follow the standards and procedures established by the US Centers for Disease Control (CDC) and are inspected biannually by the DHHS. Disclosure of donors' medical information was performed in strict compliance with HIPAA regulations for the protection of donors' privacy. We obtained approval for carrying out these studies from the National Institutes of Health (NIH) Office of Human Subjects Research Protection (OHSRP) and Biosafety Committee, Bethesda, MD, USA. All methods were performed in accordance with the guidelines and regulations approved by NIH Biosafety Committee, Bethesda, MD, USA.

Table 1 shows the demographics of the donors used for the study. AnaBios generally obtains donor organs/tissues from adults aged 18 to 60 years old. Some donors may be trauma victims, but the donor pool did not include donors with human immunodeficiency virus, donors after cardiac death, hepatitis B virus, ongoing infection, hepatitis C virus, downtime >20 min, methicillin-resistant *Staphylococcus aureus*, and positive blood cultures.

Donor DRGs (T1-T12, L1-L5, S1) from males and females were recovered using AnaBios' proprietary surgical techniques and were shipped to AnaBios via dedicated couriers. Upon arriving at AnaBios, each set of DRGs was assigned a unique identifier number that is reproduced on all

Table 1. Donor information.

Donor identifier	Age	Sex	Ethnicity	BMI	COD
Donor 1	48	F	African American	47.8	CVA/ICH/Stroke
Donor 2	27	M	Hispanic	27.6	CVA/ICH/Stroke
Donor 3	28	M	African American	29.0	Anoxia/Drug Intoxication
Donor 4	30	M	African American	25.8	Anoxia/Drug Intoxication
Donor 5	22	F	Caucasian	33.1	Anoxia/Drug Intoxication
Donor 6	48	F	African American	47.8	CVA/ICH/Stroke
Donor 7	53	M	Caucasian	31.1	Anoxia/Cardiovascular

BMI, Body Mass Index; COD, Cause of death; CVA, cerebrovascular accident; ICH, intracerebral hemorrhage.

relevant medical history files, data entry forms and electronic records.

Dissociation of human dorsal root ganglia neurons

The DRGs were then further dissected in cold proprietary neuroplegic solution to remove all connective tissue and fat. The ganglia were dissociated following the methods previously described.[23] Briefly, DRG were enzymatically digested at 37°C for 2 h using AnaBios' proprietary mixture of enzymes. After washing, the neurons were resuspended in DMEM/F12 (Lonza; Allendale, NJ) containing 1% horse serum (Thermo Fisher Scientific; Rockford, IL). Ganglia were gently triturated through the fire-polished tip of a sterile glass Pasteur pipette. Dissociated cells were plated on glass coverslips coated by poly-D-lysine. The isolated neurons put in culture in DMEM F-12 (Gemini Bio-Products CAT#: 900-955. Lot# M96R00J) supplemented with Glutamine 2 mM, Horse Serum 10% (Invitrogen #16050-130), hNGF (25 ng/ml) (Cell Signaling Technology #5221LF), GDNF (25 ng/ml) (ProSpec Protein Specialist #CYT-305) and Penicillin/Streptomycin (Thermo Fischer Scientific #15140-122).

Viral infection of human dorsal root ganglia neurons

To confirm expression of the Cdk5 activator p35 before packaging into HSV, an expression vector with an untagged human p35, pCMV-p35 (gift from Dr. Li-Huei Tsai, Addgene plasmid # 1347, Watertown, MA), was transfected into Neuro 2a cells using Neuro-2a Cell Avalanche transfection reagent (EZ Biosystems, College Park, MD). The expression of p35 was confirmed through Western blot, performed as previously described.[4] After the confirmation of p35 expression in Neuro 2a cells, pCMV-p35 was cloned into pDONR221 Gateway entry vector (Epoch Life Science; Sugar Land, TX) and packaged into a herpes simplex virus 1 (HSV-1) vector (Dr. Rachael Neve of the Gene Delivery Technology Core, Massachusetts General Hospital). Replication-deficient virus was packaged via the amplicon system and purified on a sucrose gradient. In the HSV vector, an IE4/5 promoter drives p35 expression while the mCMV promoter drives the green fluorescent protein (GFP).

The cultures were infected by either HSV-p35 or HSV-GFP, about 2 days post plating, at a multiplicity of infection (MOI) of 3. Uninfected neurons were also assessed to estimate the effects of viral infection. The neurons were used for recordings 1–4 days after infection.

Whole-cell current clamp recordings

Whole-cell patch-clamp recordings were conducted in hDRG neurons 3 to 6 days after dissociation (Figure 2(a)). The recordings were performed at room temperature (~23°C) using HEKA EPC-10 amplifier. Data were acquired on a Windows-based computer using the PatchMaster program (v2 × 90.4; HEKA Electronics). Pipettes (1.5–3.0 MΩ) (Warner Instruments #64-0792) were fabricated from 1.5 mm borosilicate capillary glass using a Sutter P-97 puller. The pipette was filled with a solution containing 110 mM K-gluconate, 20 mM KCl, 8 mM NaCl, 4 Mg-ATP, 10 mM EGTA, and 10 mM HEPES (pH 7.3 adjusted with KOH; 280 ± 5 mOsm). Neurons on Corning glass coverslips (Thomas Scientific #354086) were transferred to a RC-26GLP recording chamber (Warner Instruments #64-0236) containing 0.5 mL standard external solution containing 145 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM dextrose (pH 7.4 adjusted with NaOH; 300 ± 5 mOsm). Extracellular solution exchange was performed with rapid exchange perfusion system (flow rate 0.5–1 ml/min) (Warner Instruments #64-0186). Neurons for recordings were selected based on smoothness of the membrane. GFP control neurons and p35-transfected neurons were selected based on the positive fluorescence via GFP. Signals were filtered at 3 kHz, sampled at 10 kHz. Once whole-cell access was obtained the cell was allowed an equilibration time of at least 5 min. Neurons showing series resistance >15 MΩ were excluded from further recordings. Unless otherwise indicated, all chemicals were purchased from Sigma-Aldrich.

Measurement of rheobase

Once the cell under recording stabilized, the rheobase of single action potentials was assessed. Step currents 20 ms in duration were delivered every 10 s. Current amplitude was

increased, until action potentials can be reliably induced. The step increments were 20 pA if the current amplitude was <1000 pA and 50 pA if the current amplitude was >1000 pA. The minimum current needed for stable action potential induction was reported as rheobase. After measuring rheobase in baseline, 1 μM prostaglandin E2 and 100 nM bradykinin were applied through bath superfusion (Figure 2(b)). The rheobase current was measured after 5 minutes of exposure.

Measurements of cyclin-dependent kinase 5 activity in vitro

Different inflammatory cocktail preparations were tested first for their ability to induce Cdk5 activity in SH-SY5Y cells before use on hDRG neurons cultures. SH-SY5Y is a human neuroblastoma cell line that can be readily expanded to provide enough protein to run a CDK5 assay to measure kinase activity. SH-SY5Y cells were differentiated as previously described.[26] SH-SY5Y cells were transfected with either an empty vector or with a vector expressing His-tagged human p35 (gift from Dr. Harish Pant) using Lipofectamine 3000 (Life Technologies, Carlsbad, CA). Two days after transfection, cells were treated with various inflammatory mediators. CDK5 activity was measured as previously described.[4]

Data analysis

In addition to the resting membrane potential (RMP) and rheobase, the kinetics of single action potentials evoked by the rheobase currents were measured (Figure 2(c)).[27] Data were expressed as mean ± SEM. Kruskal Wallis test (GraphPad Prism v9.0.0) with Dunn's test multiple comparisons or Wilcoxon signed-rank test were used to determine the statistical significance ($p = 0.05$) as specified in the figure legends. The numbers in the results represent the number of neurons regardless of the donors.

Results

Cyclin-dependent kinase 5 is widely expressed in human sensory neurons and is activated by inflammatory mediators

Recent single nucleus RNAseq of human DRG neurons showed that *CDK5* and *CDK5R1*, encoding p35, are expressed in almost every transcriptomics cluster.[25] To determine the impact of CDK5 activation on the properties of human sensory neurons, we took advantage of the fact that p35 overexpression alone produces hyperalgesia in rodents. Transient overexpression of p35 has been used to examine the role of CDK5 mediated phosphorylation on both TRPV1 and TRPA1 channel activity and has not shown any of the cytotoxic effects known to be associated with p35 calpain cleavage product p25.[11,28] To achieve p35-induced activation in hDRG neurons, we first generated a p35-expressing HSV vector. The HSV provides an efficient delivery tool for expression of exogenous genes in dissociated DRG neurons.[29] After, confirming the expression of exogenous p35 by the construct in N2a cells (Figure 1(a)), the coding sequence of untagged human p35 was then packaged into HSV. This vector was used for infection of dissociated hDRG neurons.

Since inflammation of the hind paw causes increased Cdk5 activity in rodent DRG, we wanted to test if inflammatory mediators would likewise increase CDK5 activity in human neuronal cells. We first used differentiated SH-SY5Y cells as a surrogate model to examine differing combinations and concentrations of known inflammatory mediators (including bradykinin [BK], prostaglandin E_2 [PG], serotonin [5HT], and histamine) on CDK5 activity (Figure 1(b)). SH-SY5Y were transfected with either an empty plasmid or with a

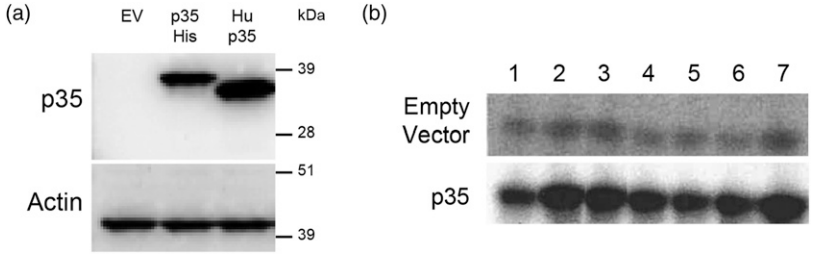

Figure 1. Expression and experimental activation of CDK5 in human neuroblastoma cell line. (a) A human p35 expression vector was transfected into N2a cells. An empty vector (EV) and a His-tagged p35 vector were used as controls. Western blot for p35 was performed before sending the expression vector to be packaged into HSV. A 35 kDa band corresponding to the untagged human p35 was detected by Western. (b) SH-SY5Y cells were differentiated and then transfected with a His-tagged p35 expression vector or an empty vector. Next, cells were treated with the following inflammatory mediators: Lane 1, non-treated; lane 2, 100 nM bradykinin (BK) + 1 μM PGE2 (PG); lane 3, 10 μM BK + 10 μM PG; lane 4, 100 nM BK + 1 μM PG + 10 μM 5HT + 10 μM histamine; lane 5, 100 nM BK + 1 μM PG + 1 mM 5HT + 1 mM histamine; lane 6, 10 μM BK + 10 μM PG + 10 μM 5HT + 10 μM histamine; lane 7, 10 μM BK + 10 μM PG + 1 mM 5HT + 1 mM histamine. CDK5/p35 was immunoprecipitated and kinase activity was evaluated by the level of phosphorylated P^{32} histone H1, a substrate of cyclin-dependent kinases.

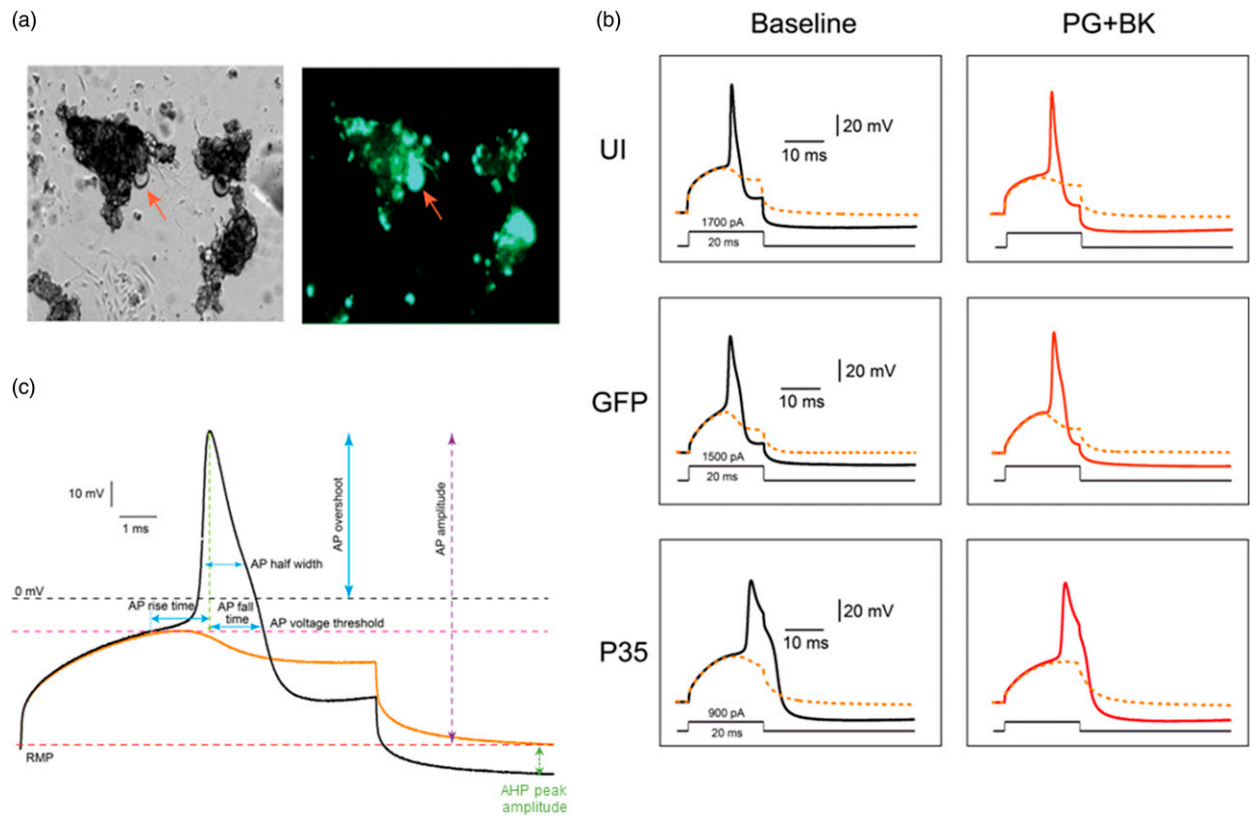

Figure 2. Action potential (AP) measurements in human dorsal root ganglia (hDRG) neurons. (a) Representative bright field image (left) and fluorescence image showing green fluorescence protein (GFP) signal (right) in dissociated hDRG neurons infected with HSV. The arrow indicates the GFP-expressing neuron recorded in patch clamp. (b) A representative AP trace (*black*) evoked at rheobase and a superimposed subthreshold trace without AP (*dashed orange trace*) in three groups (uninfected control; UI), (GFP control; GFP) and (p35 transfected; p35 hDRG neurons) before (baseline) and after perfusion with prostaglandin E2 (1 μM) and bradykinin (100 nM) (*red trace*). (c) A representative enlarged AP trace depicting AP measurements.[27] Rheobase: Minimal required threshold current to evoke an AP by an incrementing series of depolarizing pulse (incremental step; 50 pA and duration; 20 milliseconds), AP voltage subthreshold and resting membrane potential (RMP) are shown as *dashed pink line* and *dashed red line* respectively. AP rise time: AP rise to the peak (relative to AP threshold), AP amplitude: AP peak height from RMP, AP overshoot: AP amplitude from zero membrane potential (*dashed black line*), AP fall time: AP peak back to the threshold value, AHP: after hyperpolarization potential peak amplitude below RMP. AP half width: AP duration at half the AP amplitude.

His-tagged human p35 expression vector. CDK5 was then immunoprecipitated from treated SH-SY5Y cells and incubated with a fixed amount of the substrate histone H1. As shown in Figure 1(b), 100 nM BK and 1 μM PG showed robust induction of CDK5 activity, both with and without additional transfection of p35. Therefore, the mixture of these two mediators (PG/BK) was used in the following experiments.

p35 overexpression depolarizes the resting membrane potential and reduces the rheobase current in human dorsal root ganglia neurons

To determine the changes in sensory neurons excitability resulting from the activation of CDK5, we infected dissociated hDRG neurons with HSV encoding p35. Neurons infected by HSV encoding GFP served as control (Figure 2(a)). The electrophysiological properties of uninfected (UI) hDRGs were also measured to control the effects of HSV infection. Action potential firings in each neuron were assessed in baseline and after the bath application of PG/BK (Figure 2(b)). The kinetics of action potentials were analyzed as exemplified in Figure 2(c). Data were obtained from 10 UI neurons, 9 GFP-expressing neurons, and 9 p35-expressing neurons. By using whole-cell current clamp recordings (Figure 3(a)), we first assessed the RMP. The three groups showed significant differences in RMP ($p = 0.044$ in Kruskal Wallis test). In pair-wise comparisons, p35 overexpression significantly depolarized the resting membrane potential ($p = 0.038$ in Dunn's test; Figure 3(a)) compared to uninfected control groups but not to GFP group ($p = 0.86$ in Dunn's test; Figure 3(a)). Then, we assessed rheobase current by injecting progressively increasing current steps. Three groups showed significant differences in rheobase currents ($p = 0.033$ in Kruskal Wallis test). In pair-wise comparisons, the overexpression of p35 significantly reduced the rheobase current in p35 transfected neurons compared to uninfected control groups. ($p = 0.027$ in Dunn's test; Figure 3(b)). These results suggest that activation of CDK5 increases neuronal

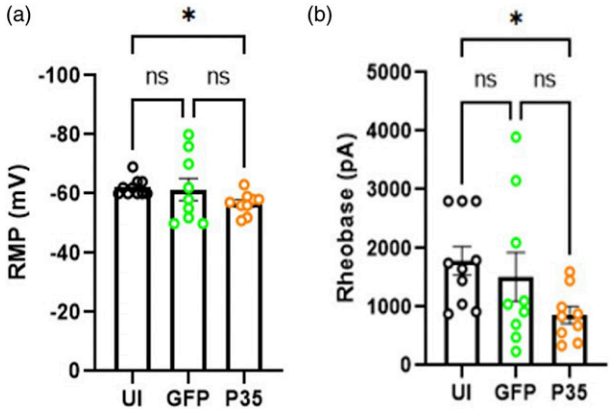

Figure 3. p35 over expression alters the RMP and rheobase current of hDRG neurons. Bar diagrams (mean ± SEM) showing RMP (a), and rheobase current (b) in neurons from UI (*n = 10; black circle*), GFP (*n = 9; green circle*) and p35 (*n = 9; orange circle)* groups. Statistical comparisons were performed using Kruskal Wallis test followed by Dunn's multiple comparisons test. *$p < 0.05$, ns (non-significant).

excitability by reducing the rheobase current and depolarizing the resting membrane potential.

p35 overexpression changes the shape of action potential in human dorsal root ganglia neurons

We determined the changes in the AP shape using the parameters described in Figure 2C. Three groups showed significant differences in AP rise time ($p = 0.003$ in Kruskal Wallis test). In posthoc pair-wise comparisons, the overexpression of p35 significantly increases the AP rise time compared to GFP ($p = 0.011$ in Dunn's test; Figure 4(a) and (c)) and uninfected control groups ($p = 0.009$ in Dunn's test; Figure 4(a)). Three groups showed significant differences in AP fall time ($p = 0.026$ in Kruskal Wallis test). In the multiple comparisons, overexpression of p35 significantly increases the AP fall time compared to GFP group ($p = 0.03$ in Dunn's posthoc test; Figures 2(b) and 4(b)). Consistently, three groups showed significantly different AP half width ($p = 0.003$ in Kruskal Wallis test), and the posthoc test showed that

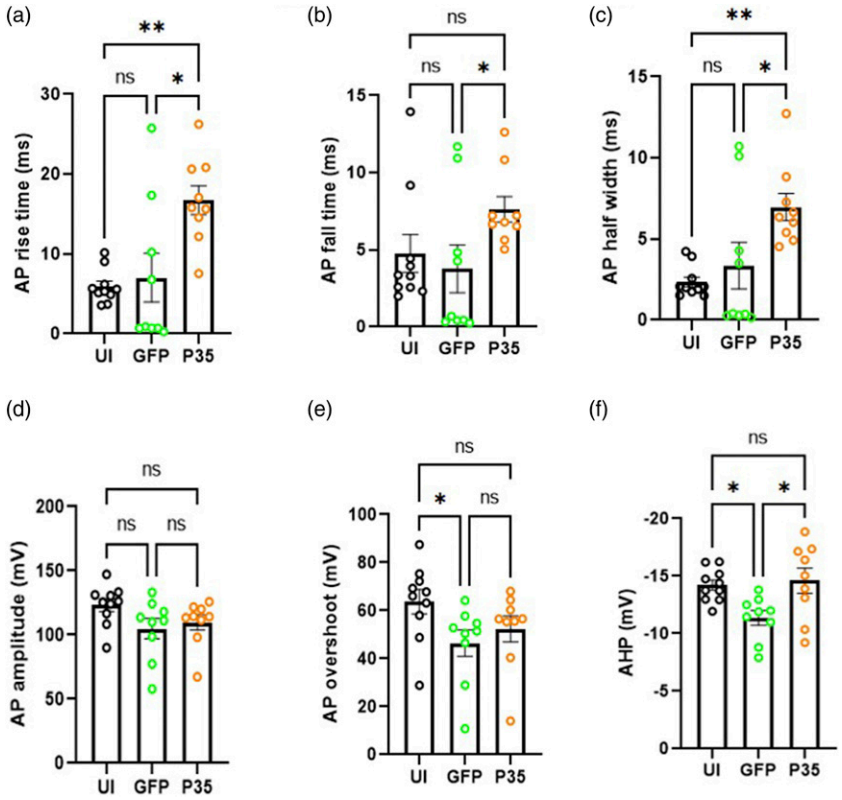

Figure 4. p35 over expression alters the AP properties in hDRG neurons. Bar diagrams (mean ± SEM) showing AP rise time (a), AP fall time (b), AP half width (c), AP amplitude (d), AP overshoot (e), and AHP (f) in neurons from UI (*n = 10; black circle*), GFP (*n = 9; green circle*) and p35 (*n = 9; orange circle)* groups. Statistical comparisons were performed using Kruskal Wallis test followed by Dunn's multiple comparisons test. *$p < 0.05$, **$p < 0.01$, ns (non-significant).

the overexpression of p35 significantly increases the AP half width compared to GFP (p = 0.011 in Dunn's test; Figure 4(c)) and uninfected control groups (p = 0.007 in Dunn's test; Figure 4(c)).

The three groups did not exhibit significant difference in AP amplitude (p = 0.067 in Kruskal Wallis test; Figure 4(d)). Although AP overshot was significantly different among the three groups (p = 0.024 in Kruskal Wallis test) posthoc pair-wise comparisons showed a significant difference only between UI and GFP (p = 0.02 in Dunn's test; Figure 4(e)). The afterhyperpolarization potential (AHP) was also different among the three groups (p = 0.01 in Kruskal Wallis test) and the posthoc test showed that AHP in the GFP group was significantly reduced compared to the UI (p = 0.04 in Dunn's test; Figure 4(f)) and the p35 groups (p = 0.02 in Dunn's test; Figure 4(f)). Overall, the changes in the shape of action potential suggest that activation of CDK5 by p35 overexpression broadens the action potential in hDRG neurons.

p35 overexpression does not enhance the responsiveness of human dorsal root ganglia neurons to prostaglandin E2 and bradykinin

Next, we investigated the effect of inflammatory mediators (PG/BK) on the electrophysiological properties of hDRG neurons in p35-transfected, GFP-transfected, and uninfected groups. After assessing RMP, rheobase currents, and AP properties under baseline condition, PG/BK was bath applied for 5 minutes and APs were evaluated again. PG/BK significantly depolarized the RMP in UI (p = 0.03 in Wilcoxon signed rank test Figure 5(a), upper panel). However, PG/BK exposure did not significantly depolarize GFP-expressing hDRGs (p = 0.14; in Wilcoxon signed rank test Figure 5(a), upper panel). Similarly, the p35 group did not show a difference either after PG/BK application (p = 0.41; in Wilcoxon signed rank test Figure 5(a), upper panel). The percent change of RMP was significantly different among the three groups (p = 0.034 in Kruskal Wallis test) but posthoc pair-wise comparisons showed no significant changes (Figure 5(a), lower panel). Next, we assessed the rheobase current before and after PG/BK exposure. PG/BK application significantly reduced the rheobase currents compared to baseline in all three groups (UI: p = 0.002; GFP: p = 0.04 and p35: p = 0.02 in Wilcoxon signed rank test; Figure 5(b), upper panel). Comparisons of percent changes of rheobase currents among three groups showed a significant difference (p = 0.023 in Kruskal Wallis test Figure 5(b), lower panel) and post-hoc pair-wise comparisons showed that p35 group showed a significantly lower change in rheobase currents in response to PG/BK compared to GFP group (p = 0.02 in Dunn's test; Figure 5(b), lower panel). PG/BK application significantly increases the AP rise time in UI (p = 0.014), but not in GFP (p = 0.3) and p35 group (p = 0.16) (Wilcoxon signed rank test; Figure 6(a), upper panel). The percentage changes of the three groups were not significantly different. Other AP parameters were not significantly affected by the PG/BK exposure in any groups (Figure 6(b)–(f)).

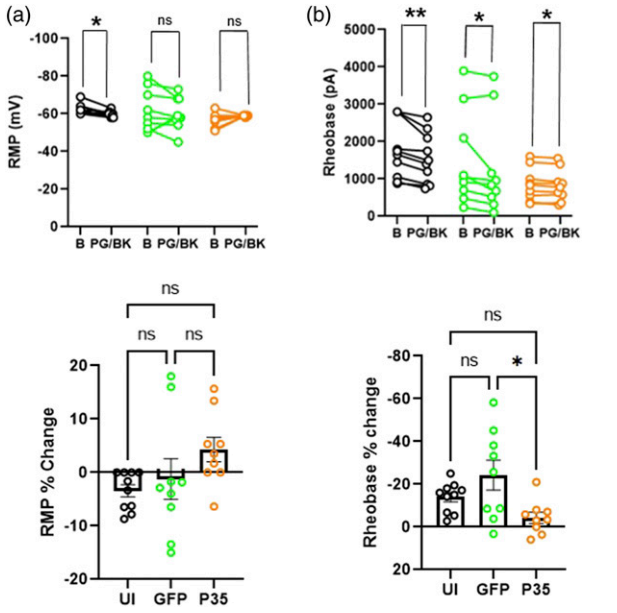

Figure 5. The effect of prostaglandin E2 (PG) and bradykinin (BK) on the RMP and rheobase current of hDRG neurons. Paired dot plot graph depicting (a) RMP, and (b) rheobase current before (baseline; (b) and after 1 μM prostaglandin E2 and 100 nM bradykinin (PG/BK) in UI (*n = 10; black circle*), GFP (*n = 9; green circle*) and p35 (*n = 9; orange circle)* neurons. In each section, the upper panel represents absolute value and lower panel represents % change. Statistical comparisons were performed using Wilcoxon signed rank test for paired data analysis and Kruskal Wallis test for percent changes. Dunn's multiple comparisons test was performed for pairwise comparisons of percent changes. *p < 0.05, **p < 0.01, ns (non-significant).

Discussion

The contribution of CDK5 in primary afferents has been well established in preclinical rodent models. However, there is no evidence supporting the roles of CDK5 in nociceptor functions in humans. In this study, we have used an electrophysiological method to investigate the role of CDK5 on the excitability and action potential properties of hDRG neurons in culture by the overexpression of p35. Due to the technical limitation, we were not able to directly determine the increased CDK5 activities by p35 overexpression in human DRG neurons, However, since p35 is a well-established specific activator of CDK5, we interpret our data as the function of CDK5. To the best of our knowledge, this is the first report demonstrating that the activation of CDK5 influences the AP properties in human sensory neurons. In this study, we elected to test two pathologically relevant conditions to determine the impact of CDK5 activation on the properties of hDRG neurons. First, we mimicked the

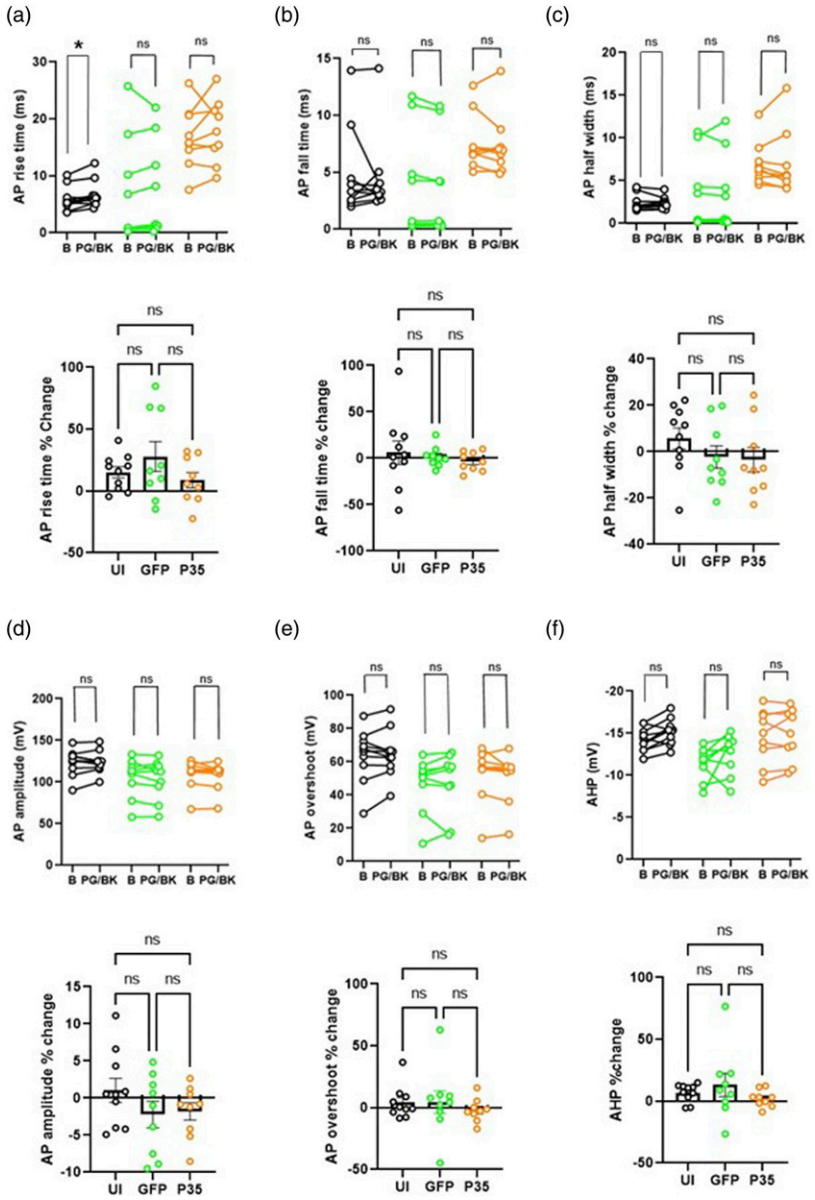

Figure 6. The effect of prostaglandin E2 (PG) and bradykinin (BK) on the AP properties of hDRG neurons. Paired dot plot graph depicting (a) AP rise time, (b) AP fall time, (c) AP half width, (d) AP amplitude, (e) AP overshot, (f) AHP before (baseline; (b) and after 1 μM prostaglandin E2 and 100 nM bradykinin (PG/BK) in UI (*n = 10; black circle*), GFP (*n = 9; green circle*) and p35 (*n = 9; orange circle)* neurons. In each section, the upper panel represents absolute value and lower panel represents % change. Statistical comparisons were performed using Wilcoxon signed rank test for paired data analysis and Kruskal Wallis test for percent changes. Dunn's multiple comparisons test was performed for pairwise comparisons of percent changes. *$p < .05$, **$p < .01$, ns (non-significant).

upregulation of CDK5 activity by the increased expression of p35. In mice, overexpression of p35 alone increases pain sensitivity, even in the absence of inflammation or nerve injury,[39] suggesting that p35 overexpression is sufficient to produce hyperalgesia. Our hDRGs culture model replicated this condition by inducing exogenous p35 overexpression following infection with an HSV vector carrying the p35 gene. Second, we tested the effect of nociceptor sensitization by inflammatory mediators on CDK5. Our data support the activation of CDK5 by PG/BK in hDRGs. HSV infection led to enhanced excitability of hDRG neurons, manifested as reduction of the rheobase current and RMP. However, no significant difference was observed between p35-and GFP-overexpressing neurons, suggesting that these differences are not related to p35 specifically but to the viral infection. On the contrary, p35 overexpression altered the AP rise time, AP fall time and AP half-width leading to AP broadening. The impact of p35 overexpression on the broadening of AP strongly supports the idea that CDK5 activation influences the electrophysiological properties of hDRG neurons.

Acute application of PG/BK altered the membrane properties (rheobase and RMP) and AP properties (AP rise time) in uninfected hDRG neurons. This is consistent with the fact that inflammation enhances Cdk5 activity in DRG of rodents.[30] However, PG/BK application did not produce any significant changes either in membrane properties or AP properties in GFP- and p35-expressing groups, and hence we do not have evidence that p35 overexpression alters responses to inflammatory mediators in human DRG neurons. Given the tendency of depolarized membrane potential and decreased rheobase following p35 HSV infection, we postulate that additional activation of CDK5 by PG/BK following p35 overexpression may be modest and does not produce any further changes. Again, however, the interpretation of the data is limited by the large variation of the data, the impact of HSV infection, and the insufficient sample size. The inability of targeting a specific subpopulation of neurons, e.g., nociceptors, could have been a contributor to the increased variation of the results.

AP kinetics represents the ensemble of depolarizing and repolarizing ionic conductances. Intricate balances of voltage-gated sodium, calcium, and potassium channels produce neuronal AP firing. Different inward currents from TTX-sensitive and TTX-resistant Na^+ channels and Ca^{2+} currents contribute to different phases of AP in DRG neurons.[31] Action potential of nociceptive neurons shows wide action potential with a characteristic hump on the falling phase.[32–36] Such characteristic is attributable to TTX-resistant Na^+ channels and voltage-gated Ca^{2+} channels. Large conductance Ca^{2+}-activated K^+ channels also contribute to the duration of AP in non-peptidergic nociceptive DRG neurons.[37] Since the observed changes in AP shape are consistent with upregulation of TTX-R Nav1.8, and the biophysical properties of this channel (fast recovery from inactivation) enable nociceptive neurons to sustain significantly higher firing rates, the AP shape changes may lead to encoding higher pain intensity. We also speculate that AP broadening should increase Ca^{2+} influx in presynaptic terminals, which can induce more efficient nociceptive synaptic transmissions. The peripheral and central nociceptive system may be optimized for the low-frequency encoding in the transduction and conduction of nociceptive signal to differentiate it from the high-frequency non-nociceptive conduction systems, such as tactile sensation or proprioception.

Importantly, AP width is not static, but the broadening of AP in sensory neurons occurs in multiple pathological conditions through various mechanisms. For example, monocyte chemoattractant protein-1 broadens action potential in DRG neurons through the increase of non-selective cationic currents and the inhibition of voltage-dependent K^+ currents.[38] Chronic constriction injury of the infraorbital nerve or chemotherapy-induced neuropathy by oxaliplatin leads to an increase in AP widths of trigeminal ganglia neurons, which is likely through the downregulation of Kv4.3.[5,39] Peripheral axotomy broadens AP mediated by the downregulation of Kv2.1 and Kv2.2.[40] Mutation of Nav1.7 associated with erythromelalgia increases AP width.[41] Our study shows that the activation of CDK5 through p35 overexpression broadens AP in human DRG neurons. Since Cdk5 is upregulated in the DRG after spinal nerve ligation in rats,[15] AP broadening can be associated with the pathological condition likely contributing to neuropathic pain in humans. Therefore, our data strongly suggest that CDK5 can play a modulatory role in human primary afferents contributing to chronic pain.

Since we did not evaluate CDK5-induced changes in ionic currents by voltage clamp, we do not have evidence underlying the mechanisms of AP broadening. AP width is predominately determined by Ca^{2+} transients, potassium, and sodium channel activities.[42–44] The inhibition of Cdk5 activity reduces the TRPV1-mediated Ca^{2+} influx in rat DRG neurons.[3] Nav1.7 channel activity is regulated by CDK5-dependent phosphorylation of CRMP2 (a binding partner of NaV1.7) in neuropathic pain condition.[45] Cdk5 also phosphorylates and regulates both high voltage-activated N-type Cav2.2[46] and low voltage-activated T-type Cav3.2[47] Ca^{2+} channels. CDK5 regulates the voltage-gated Kv2.1 channels through direct phosphorylation, which is crucial to activity-dependent plasticity in intrinsic neuronal excitability.[48] CDK5 also regulates the activity of Kv7.2 channels through its phosphorylation.[49] Our substrate analysis suggested that CDK5 may phosphorylate other voltage-gated ion channels, such as Nav1.9, Cav3.3, Kv6.2, and $KCa^{2.1}$ [12]. Therefore, CDK5 activation may broaden AP in hDRG through the ensemble regulation of the voltage-gated ion channels, which suggests that CDK5 is an important upstream regulator of AP properties in pathological conditions.

In conclusion, CDK5 activation through the exogenous overexpression of p35 in dissociated hDRG neurons broadens AP. Our data suggest that CDK5 plays an important role in the modulation of AP properties in human primary afferents under the conditions in which CDK5 is upregulated, contributing to chronic pain.

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ORCID iDs

Man-Kyo Chung https://orcid.org/0000-0001-7637-1148
Ashok B Kulkarni https://orcid.org/0000-0001-9849-3250

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