

# Identification of Thr<sup>283</sup> as a key determinant of P2X<sub>7</sub> receptor function

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**Short title:** mouse P2X<sub>7</sub> polymorphisms

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**Abbreviations:** ATP, adenosine 5'-triphosphate; BzATP, 2'-3'-O-(4-benzoylbenzoyl) adenosine 5'-triphosphate; HEK293, human embryonic kidney cell line; EtBr, ethidium bromide; PVDF, polyvinylidene difluoride

## Abstract

**Background and purpose:** The ATP-gated P2X7 receptor is an unusual ion channel that couples to multiple downstream signalling cascades. We noted differences in mouse cDNA sequences that may indicate polymorphisms; the aim of this study was to compare function and expression of these mouse P2X7 receptor mutations.

**Experimental approach:** There are three differences in the sequences of P2X7 cDNA cloned from mouse NTW8 microglial cells or C57 BL/6 mice: [Phe<sup>11</sup>,Ala<sup>221</sup>,Met<sup>283</sup>]P2X7 in the former and [Leu<sup>11</sup>,Thr<sup>221</sup>,Thr<sup>283</sup>]P2X7 in the latter. We expressed these receptors and measured membrane currents, ethidium uptake, calcium influx and surface membrane expression. We also carried out these assays on the previously described polymorphism observed between C57 BL/6 and Balb/c mice ([Leu<sup>451</sup>]P2X7 vs [Pro<sup>451</sup>]P2X7).

**Key results:** Maximum current densities at [Phe<sup>11</sup>,Ala<sup>221</sup>,Met<sup>283</sup>]P2X7 were <12% of those at [Leu<sup>11</sup>,Thr<sup>221</sup>,Thr<sup>283</sup>]P2X7 without change in the agonist concentration-response. Replacing methionine with threonine at residue 283 yielded a receptor whose properties were the same as [Leu<sup>11</sup>,Thr<sup>221</sup>,Thr<sup>283</sup>]P2X7. Replacing T<sup>283</sup> in the rat P2X7 receptor with methionine yielded currents that were <10% of wildtype and no ethidium uptake was associated with its activation. Maximum current densities and agonist EC<sub>50</sub> values were the same at mouse [Thr<sup>283</sup>,Leu<sup>451</sup>]P2X7 and [Thr<sup>283</sup>,Pro<sup>451</sup>]P2X7 but ethidium uptake and Fluo4

fluorescence were significantly reduced at the [Thr<sup>283</sup>,Leu<sup>451</sup>]P2X<sub>7</sub> receptor.

There was equivalent surface membrane expression of all P2X<sub>7</sub> receptors.

**Conclusions:** This study has revealed a residue (Thr<sup>283</sup>) in the ectodomain that is critical for P2X<sub>7</sub> receptor function and suggests that the intracellular residue 451 alters downstream signalling independently of ion channel activity.

## Introduction

P2X<sub>7</sub> receptors are ATP-gated ion channels expressed primarily in cells of immune function, such as monocytes, macrophages and brain microglia and astrocytes (North, 2002). In these cells and in mammalian expression systems, their activation results in the opening of non-selective cation channels which are highly permeable to calcium (North, 2002). Uniquely for P2X<sub>7</sub> receptors, activation also leads to opening of large dye-permeable pores, rapid membrane and cytoskeletal rearrangement and release of pro-inflammatory cytokines. In the case of prolonged or repeated stimulation, cell death occurs via necrotic or apoptotic pathways (Di Virgilio *et al.*, 2001; Elliott *et al.*, 2005; Guerra *et al.*, 2003; Le Feuvre *et al.*, 2002; Le Feuvre *et al.*, 2003; MacKenzie *et al.*, 2001; Mackenzie *et al.*, 2005; Pfeiffer *et al.*, 2004; Rothwell, 2003; Suh *et al.*, 2001; Verhoef *et al.*, 2003). Moreover, P2X<sub>7</sub> receptors are rapidly up-regulated and activated after initial inflammatory stimuli (Franke *et al.*, 2004; Verhoef *et al.*, 2003). P2X<sub>7</sub> receptors are therefore promising targets in terms of treatment of

inflammatory diseases such as arthritis, and the after-effects of cerebral ischaemia (Alcaraz *et al.*, 2003; Baxter *et al.*, 2003; Guerra *et al.*, 2003; Rothwell, 2003).

Characterisation of P2X<sub>7</sub> receptor knock-out mice highlights the importance of this receptor in the inflammatory response. These mice display altered leukocyte function, cytokine production and inhibition of chronic inflammatory and neuropathic pain (Chessell *et al.*, 2005; Labasi *et al.*, 2002; Solle *et al.*, 2001). Availability of the knock-out mouse provides a useful tool to study the function of P2X<sub>7</sub> receptors, particularly as there are currently no readily available antagonists selective for this receptor. However, in contrast to numerous studies carried out on human and rat P2X<sub>7</sub> receptors expressed in mammalian cells (North, 2002), relatively few studies have examined the properties of mouse P2X<sub>7</sub> receptor (Adriouch *et al.*, 2002; Chessell *et al.*, 1998b; Hibell *et al.*, 2000). Because significant species differences in the pharmacology between human and rat P2X<sub>7</sub> receptors exist (Bianchi *et al.*, 1999; Chessell *et al.*, 1998a; Hibell *et al.*, 2001; Humphreys *et al.*, 1998; Virginio *et al.*, 1997), the lack of information on the physiological and pharmacological properties of mouse P2X<sub>7</sub> receptors expressed in isolation presents obstacles toward accurate interpretations of data obtained from P2X<sub>7</sub> receptor wildtype and knockout mice studies. Further, there is at least one single nucleotide polymorphism (P451L) in mice, but not apparently in humans, which is known to have significant functional consequences (Adriouch *et al.*, 2002). We also noted three other single amino

acid differences (F11L, A221T and M283T) in the published sequences from the original mouse cDNA cloned from NTW8 microglial cells (Chessell *et al.*, 1998b) and those cloned from C57 BL/6 and Balb/c mice (Adriouch *et al.*, 2002) which may indicate further polymorphisms.

The aim of the present study was to provide a functional characterization of these mouse P2X<sub>7</sub> receptor mutations and a direct comparison to the properties of the rat P2X<sub>7</sub> receptor expressed in HEK cells and studied under identical conditions. We measured membrane currents, ethidium uptake and cytoplasmic calcium changes using whole-cell recordings and single-cell fluorescence assays, and examined surface membrane expression of the receptors semi-quantitatively using Western blotting and biotinylation assays. All receptors were similarly expressed at the plasma membrane. The mutations at residues 11 and 221 (F11L, A221T) did not alter receptor function but the presence of the methionine at residue 283 resulted in markedly reduced channel activity of either mouse or rat P2X<sub>7</sub> receptor. We also found that the P451L mutation resulted in decreased cytoplasmic calcium increases and ethidium uptake, as previously described (Adriouch *et al.*, 2002), but this was not associated with decreased membrane expression or agonist-evoked membrane currents.

## Methods

### *Chemicals and antibodies*

Culture media, sera and other cell culture reagents were obtained from Life Technologies (Paisley, UK). Ethidium bromide (EtBr), ATP, BzATP, pluronic acid, Triton-X-100 and Kodak film were obtained from Sigma (Poole, UK). Fluo-4 AM was obtained from Molecular Probes (Eugene, Oregon), anti-Glu-Glu antibody from Bethyl Laboratories (Cambridge, UK), goat anti-rabbit HRP-conjugated secondary antibody from DAKOCytomation, (Ely, UK) and ECL-plus kit from Amersham (Bucks, UK).

### *Cell culture, transfection and site-directed mutagenesis*

HEK293 cells were transiently transfected using Lipofectamine 2000 (Invitrogen). Cells were plated either into 60mm dishes (cell surface labelling) or onto 13 mm glass coverslips (functional studies) and maintained in Dulbecco's modified Eagle medium, supplemented with 10% heat inactivated fetal calf serum and 2mM L-glutamine at 37°C in a humidified 5% CO<sub>2</sub> incubator. Point mutations were introduced into rat or mouse P2X<sub>7</sub> receptors (Surprenant *et al.*, 1996; Chessell *et al.*, 1998b) using the PCR overlap extension method (Urban *et al.*, 1997) and Accuzyme proof-reading DNA polymerase (Bioline, London, UK). Both rat and mouse P2X<sub>7</sub> constructs were sub-cloned in the same expression vector background (pcDNA3, Invitrogen) and bore C-terminal Glu-Glu epitope tags

(EYMPME) for detection in Western blotting. The 3'-non-coding region of the mouse P2X<sub>7</sub> construct was engineered to be identical to that of the rat P2X<sub>7</sub> construct in order to eliminate any differences in expression due to non-coding sequences. The full coding regions of all constructs used in this study were verified by DNA sequencing.

### *Electrophysiological recordings*

Whole-cell recordings were made 24-48 hours after transfection using an EPC9 patch clamp amplifier (HEKA Elektronik, Lambrecht, Germany). Membrane potential was held at -60mV. Recording pipettes (5-7 M $\Omega$ ) were pulled from borosilicate glass (World Precision Instruments, Sarasota, Florida) and filled with an intracellular solution that consisted of (in mM): 145 NaCl, 10 EGTA, 10 HEPES. The external solution contained (in mM): 147 NaCl, 10 HEPES, 13 glucose, 2 KCl, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>. Agonists were applied in solution containing low divalent cation concentrations which consisted of (in mM): 147 NaCl, 10 HEPES, 13 glucose, 2 KCl, 0.2 CaCl<sub>2</sub>. Osmolarity and pH values were 295-310 mOsm/l and 7.3 respectively. All experiments were performed at room temperature. Agonists were applied using a RSC 200 fast-flow delivery system (BioLogic Science Instruments, Grenoble, France). Agonists were applied for 5- or 10-second duration to obtain steady-state responses. Concentration-response curves to ATP and BzATP were obtained by first obtaining a maximum response to agonist (marked run-up of response was observed at both rat and some

mouse P2X<sub>7</sub> receptors, (Chessell *et al.*, 1998b) and then either applying decreasing or increasing concentrations. In either case, similar curves were obtained, provided that a maximum response had been obtained beforehand. Concentration-response curves were plotted using KaleidaGraph (Synergy Software, Reading, Pennsylvania) and Prism v3.0a Software (Graphpad Prism; [www.graphpad.com](http://www.graphpad.com)) using the Hill equation provided in Prism.

#### *Cell-surface protein labelling by impermeant biotin*

Cell-surface protein was labelled with impermeant biotin using EZ-link (Pierce, Cramlington, UK). Confluent cells (two 60 mm culture dishes) were washed twice in PBS, and 0.5mg/ml EZ-link Sulfo-NHS-SS-biotin was added for 30 minutes at room temperature. Glycine was added to quench the reactions, cells were removed from the culture dishes and pelleted. Cell pellets were lysed in PBS containing 1% Triton X-100 and antiproteases (Complete, Roche, Lewes, UK) for 1 hour at 4°C, followed by centrifugation at 16000 x g for 10 minutes to pellet debris. Total protein samples were removed and assayed for protein content using the Bio-Rad Protein Assay kit (Bio-Rad, Hemel Hempstead, UK). Biotinylated surface protein in the cell lysate was bound to Immuno-pure immobilized streptavidin beads by rotating overnight at 4°C. Beads were washed three times in lysis buffer, SDS-PAGE sample buffer was added and the samples were boiled for 5 minutes at 100°C to release purified cell-surface proteins. Samples were separated on 8% polyacrylamide gels according to standard

methods (Laemmli, 1970) and transferred to PVDF membranes. Western blotting was performed according to standard protocols and proteins were visualised using anti Glu-Glu primary antibody and HRP-conjugated secondary antibody (both at 1:2000 dilution), followed by detection using the ECL-plus kit and Kodak Bio-Max MS film. Gels were analysed by densitometry measurements using NIH ImageJ (<http://rsb.info.nih.gov/ij/>) and GeneSnap/GeneTools software (Syngene).

#### *Fluo4 and dye uptake assays*

Changes in free intracellular calcium concentration were measured with the fluorescent indicator Fluo-4 AM. A Nikon confocal microscope with Fluar 20X objective (Nikon, Surrey, UK) was used. Cells plated onto 13 mm coverslips were loaded for 45 min at 37°C with 1  $\mu$ M Fluo-4AM and 0.06% pluronic acid. Cells were superfused (2 ml/min) with the same solution used for whole-cell recordings and agonist was applied by gravity to the superfusion fluid. Images were acquired at 4 s intervals; 20 cells in the field of view were measured for 200 s in each experiment and then averaged to obtain the mean fluorescence signal. The same protocol was used to measure ethidium uptake (Gu *et al.*, 2000) with ethidium bromide (25  $\mu$ M) in superfusion solutions throughout. All experiments were carried out at room temperature.

## **Results**

### *Thr-283 is critical for P2X<sub>7</sub> receptor function*

We first compared ATP and BzATP-evoked responses in cells expressing the initially cloned mouse P2X<sub>7</sub> receptor (Chessell *et al.*, 1998b) with those expressing the mouse P2X<sub>7</sub> receptor cloned from C57 BL/6 mice (Adriouch *et al.*, 2002), the strain used to derive the P2X<sub>7</sub> receptor gene-deleted mice (Chessell *et al.*, 2005; Labasi *et al.*, 2002; Solle *et al.*, 2001). These cDNAs differed by three residues: F11, A221 and M283 in the former and L11, T221 and T283 in the latter while both carry the L451 polymorphism described by Adriouch *et al.* (2002). Figure 1a shows typical currents in response to maximum concentrations of agonists at each of these receptors; current amplitudes from mouse P2X<sub>7</sub>-F11/A221/M283 were less than 12% of those recorded from mouse P2X<sub>7</sub>-L11/T221/T283 ( $49 \pm 8$  pA/pF,  $n = 11$  vs  $427 \pm 31$  pA/pF,  $n = 10$  for BzATP;  $50 \pm 6$  pA/pF vs  $386 \pm 39$  pA/pF for ATP). ATP and BzATP concentration-response curves were obtained for membrane currents evoked at -60 mV (Figure 1c). There were no significant differences in EC<sub>50</sub> values or Hill slopes obtained in spite of the striking differences in peak amplitude of agonist-evoked currents (Figure 1c). These experiments were carried out with low divalent cations (zero magnesium, 0.2 mM calcium) in order to rule out possible differences due to extracellular cation effects (Virginio *et al.*, 1997). When normal divalent cations were present we did not detect currents in response to ATP (10 mM) or BzATP (1 mM) at the mouse P2X<sub>7</sub>-F11/A221/M283 receptor ( $n=15$ ) while currents  $> 1 - 3$  nA were recorded from cells expressing mouse P2X<sub>7</sub>-

F11/A221/T283 ( $n = 8$ ). At the mouse P2X<sub>7</sub>-M283 receptor, no ethidium uptake was observed in response to maximum agonist application (in low extracellular divalent cations), in contrast to the strong signal recorded from cells expressing the mouse P2X<sub>7</sub>-L11/T221/T283 receptor (Figure 2a). The lack of functional expression of the mouse P2X<sub>7</sub>-F11/A221/M283 receptor was not caused by the L451 mutation because no differences in agonist evoked currents or ethidium uptake were observed when L451 was changed to a proline (see below).

When we replaced the methionine at residue 283 with threonine (P2X<sub>7</sub>-F11/A221/T283) maximum amplitudes to ATP and BzATP, kinetics of the currents and concentration-response curves were the same as those at the mouse P2X<sub>7</sub>-L11/T221/T283 receptor (Figure 1a,b, 3b), as was the ATP-evoked ethidium uptake (Fig 2a). Thus, it is the methionine mutation at residue 283 that renders this P2X<sub>7</sub> receptor poorly functional without involvement of the mutations at residues 11 and 221. Because all other mammalian P2X<sub>7</sub> receptors carry threonine at residue 283, we asked whether this was, indeed, critical to function of these receptors. Replacing T283 with methionine in the rat P2X<sub>7</sub> receptor reduced agonist-evoked currents by more than 90% (Figure 1b,d) and eliminated all ethidium uptake (Figure 2b). Co-expression of mouse P2X<sub>7</sub>-F11/A221/M283 receptor with mouse P2X<sub>7</sub>-L11/T221/T283 receptor did not alter the agonist-evoked ethidium uptake (data not shown), indicating that the M283 mutation was unable to act as a dominant negative subunit to reduce the function of the P2X<sub>7</sub>-T283 receptor.

Agonist concentration-current curves for rat P2X<sub>7</sub> receptors were also obtained in order to provide a direct comparison to the mouse P2X<sub>7</sub> receptors; the EC<sub>50</sub> value for ATP at mouse P2X<sub>7</sub> receptor (900 μM) was 10-fold higher than at rat P2X<sub>7</sub> receptors (90 μM) while BzATP values differed by more than 100-fold (3 μM vs 300 μM at rat and mouse respectively) (Figure 1c,d).

#### *Surface expression of mouse P2X<sub>7</sub> receptor mutants*

We next asked whether the lack of function of mouse P2X<sub>7</sub>-M283 receptors results from defective surface membrane expression, using cell-surface biotinylation followed by purification of biotinylated protein on streptavidin beads. We also compared surface expression of rat P2X<sub>7</sub> receptor (T283 wildtype) transfected at decreasing plasmid concentrations with rat and mouse P2X<sub>7</sub>-M283 and mouse P2X<sub>7</sub>-T283 receptors (Figure 3a,c). Comparable levels of both total protein and surface expression were obtained for rat and mouse P2X<sub>7</sub> when 0.1 μg/ml rat P2X<sub>7</sub> plasmid and 1 μg/ml mouse P2X<sub>7</sub> plasmid were transfected (Figure 3c). Similar levels of both total and surface protein expression were observed for mouse P2X<sub>7</sub>-M283 and T283. Figure 3b shows the relative current densities obtained for each construct in response to maximum ATP concentrations (3 mM and 10 mM at rat and mouse receptors respectively). At both rat and mouse orthologues, P2X<sub>7</sub>-M283 receptor passed minimal current, yet showed comparable levels of surface expression to P2X<sub>7</sub>-T283 receptor.

These results indicate that the lack of function of mouse or rat P2X<sub>7</sub>-M283 is not due to impaired presentation of the receptor at the cell surface.

*Mouse P2X<sub>7</sub>-P451L mutation reduces dye uptake and calcium increases independently of membrane currents*

All of the above experiments were carried out on P2X<sub>7</sub> receptors carrying leucine at residue 451 but previous studies have identified a single nucleotide polymorphism at this site (proline in Balb/c and leucine in C57 BL/6 mice; Adriouch *et al.*, 2002). The mouse P451L polymorphism was previously found to reduce both dye-uptake and cytoplasmic calcium increases in response to ATP or BzATP stimulation (Adriouch *et al.*, 2002) but neither membrane currents nor surface expression of the receptor was determined. Therefore, we examine total and surface expression of P451 and L451 at mouse P2X<sub>7</sub>-T283 receptor, and compared membrane currents, ethidium uptake and calcium influx (Figures 4,5). For these experiments, we used mouse P2X<sub>7</sub>-L11/T221/T283 receptors differing only at the P451L site; these sequences correspond to those from Balb/c (P451) and C57 BL/6 (L451) mice (Adriouch *et al.*, 2002). We found no consistent or significant differences in surface expression relative to total protein (Figure 4c), maximum agonist-evoked currents (Figure 4a,b), or agonist-evoked concentration-response curves (Figure 4d, see also Figure 1c). In contrast, consistent with the previous study of this polymorphism, we found that both

ethidium uptake (Figure 5a) and intracellular calcium concentration increases (Figure 5b) were significantly reduced in cells expressing P2X<sub>7</sub>-L451 receptors.

## Discussion

There are two significant findings from the present study; firstly, we have identified threonine-283 as a critical residue mediating P2X<sub>7</sub> receptor channel activity and secondly, the P451L polymorphism in the mouse P2X<sub>7</sub> receptor appears to inhibit downstream signalling independently of ion channel activation.

The original mouse P2X<sub>7</sub> receptor cDNA, carrying the M283 mutation, was cloned from an NTW8 mouse microglial cell line (Chessell *et al.*, 1998b); however, all published ESTs and sequenced mouse strains (as well as human and rat) have threonine in this position. There are three possible explanations for this discrepancy: (i) this mutation represents a polymorphism in the strain from which the NTW8 cell line was generated, (ii) it was generated during the NTW8 cell line generation or (iii) it was randomly generated during the cloning process. We do not have a definitive answer because the report describing the generation of this microglial cell line does not mention the mouse strain used (Chessell *et al.*, 1997) and this particular NTW8 cell line is no longer available. Irrespective of the underlying discrepancy, the threonine at position 283 is clearly of critical importance to ion channel activity at mammalian P2X<sub>7</sub> receptors. Mouse or rat

P2X<sub>7</sub>-M283 receptors showed no dye uptake to ATP or BzATP and agonist-evoked currents were 90% less than at P2X<sub>7</sub>-T283 receptors irrespective of whether the P451L mutation was also present. We also did not observe 'runup' of the response to repeated agonist application, which is often observed at the rat P2X<sub>7</sub> receptor (Surprenant *et al.* 1996; Hibell *et al.* 2000) and which was pronounced in our experiments on the mouse P2X<sub>7</sub>-L11/T221/T283 and P2X<sub>7</sub>-F11/A221/T283 receptor. The reduction in receptor activation was not due to reduced surface expression of the protein and was not associated with any change in ATP or BzATP agonist affinity. Our data are therefore more consistent with an alteration in the gating of the channel, or in formation of the signalling complex (see below) rather than with an effect at an agonist binding site or dysfunctional protein trafficking. The T283 site, although conserved in mammalian P2X<sub>7</sub> receptors, is not a conserved residue in the other six members of the P2X receptor family; it is also not associated with regions of the ectodomain that have been implicated in agonist binding based on mutagenesis experiments on P2X<sub>1</sub> and P2X<sub>2</sub> receptors (North, 2002; Vial *et al.*, 2004).

Our results are in striking contrast to the study by the Chessell group in which the P2X<sub>7</sub>-F11/A221/M283 receptor currents were large (maximum amplitudes > 2- 5 nA) with pronounced 'runup' of the current and equally pronounced dye uptake (Chessell *et al.*, 1998b). The minimal methodological differences are unlikely to account significantly for the differences between our two studies. It is clear that P2X<sub>7</sub> receptors exist in a signalling complex involving

protein-protein association with several cytoplasmic and membrane-bound signalling molecules (Adinolfi *et al.*, 2003; Donnelly-Roberts *et al.*, 2004; Kim *et al.*, 2001; Wilson *et al.*, 2002) and the rat and human P2X<sub>7</sub> receptor have been shown to be negatively regulated by diverse mechanisms involving some of these interacting proteins. For example, high levels of tyrosine phosphatase activity dramatically reduce P2X<sub>7</sub> receptor-mediated currents with pronounced 'rundown' of responses (Kim *et al.*, 2001), tyrosine phosphorylated Hsp-90 inhibits P2X<sub>7</sub> receptor currents (Adinolfi *et al.*, 2003), and p38 MAP kinase activity is directly involved in P2X<sub>7</sub>-receptor mediated dye uptake (Donnelly-Roberts *et al.*, 2004). In view of the equivalent surface membrane expression of mouse P2X<sub>7</sub>-M283 and P2X<sub>7</sub>-T283 protein, it is possible that the lack of functional expression of mouse or rat P2X<sub>7</sub>-M283 in our study results from misdirected association of one or more proteins involved in the P2X<sub>7</sub> receptor complex. This possibility is further supported by the finding that the pharmacological profile of ATP and BzATP-activated currents we obtained at the mouse P2X<sub>7</sub>-T283 receptor (EC<sub>50</sub> values of 900 μM and 300 μM for ATP and BzATP respectively) are very similar to those obtained by the Chessell group (Chessell *et al.*, 1998b).

The mouse P2X<sub>7</sub>-P451L polymorphism was initially identified based on studies of T-lymphocyte function in different strains of mice: those from C57 BL/6 or DBA/2 mice in which P2X<sub>7</sub> receptor (L451) responses (ATP-mediated calcium flux, dye-uptake, annexin-V binding and cell death) were markedly attenuated

compared to T-lymphocytes from Balb/c mice, the most commonly used strain derived from wild mice (Adriouch *et al.*, 2002). We also found reduced dye uptake and calcium flux in cells expressing the mouse P2X<sub>7</sub>-T283/L451 receptor but this was not associated with reduced surface protein expression, altered agonist concentration-response curves, or maximum current densities to agonist application when compared to mouse P2X<sub>7</sub>-T283/P451 receptors. These results would be difficult to reconcile with previously held notions that the P2X<sub>7</sub> ion channel itself provides the dye-uptake pathway, ie. that the small cationic ion channel dilates over time into a 'large pore' able to pass molecules up to 800-900Da (Virginio *et al.*, 1999a; Virginio *et al.*, 1999b). However, recent studies have provided strong, although indirect, evidence that it is not the P2X<sub>7</sub> receptor channel itself that changes conformation to become the 'large pore' but rather that its activation signals a distinct protein (or proteins) responsible for passage of larger weight molecules (Faria *et al.*, 2005; Jiang *et al.*, 2005). Our data fit more reasonably into this newer interpretation of P2X<sub>7</sub> receptor function and implicate the residue at 451 as a potential site of interaction with one, or more, proteins that are known to form a P2X<sub>7</sub> receptor signalling complex (Donnelly-Roberts *et al.*, 2004; Kim *et al.*, 2001; Wilson *et al.*, 2002). Our results also have further practical implications in terms of functional studies on P2X<sub>7</sub>-receptor knockout mice. The currently available P2X<sub>7</sub>-deleted transgenic mice have been generated from in the C57 BL/6 background (Sikora *et al.*, 1999; Solle *et al.*, 2001) and it has already been pointed out that the P2X<sub>7</sub> receptor-deficient

phenotype may be underestimated due to the already attenuated phenotype of the P451L polymorphism in the wild-type mouse (Adriouch *et al.*, 2002). Our results suggest that while this will be the case for downstream signalling processes, it may not hold for studies examining the initial membrane current due to receptor activation. Thus, further combined studies comparing P2X<sub>7</sub> receptor-activated currents and more downstream events using macrophage from mice bearing the P451 and L451 polymorphism may allow a clearer elucidation of the mechanisms leading from channel opening to 'large pore' formation and the consequences of this signalling event.

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## Figure Legends

**Figure 1** Presence of a threonine at position 283 is critical for P2X<sub>7</sub> receptor function. (a,b) Representative currents recorded in response to maximum concentrations of ATP or BzATP (as indicated by bars) at mouse (a) and rat (b) P2X<sub>7</sub>-M283/L451 or P2X<sub>7</sub>-T283/L451 receptor. (c) BzATP and ATP concentration-response curves obtained at mouse P2X<sub>7</sub> receptors; results are plotted as current densities, lines are best fit to Hill equation with each mean showing mean ± s.e. mean of 3 – 8 experiments. Calculated EC<sub>50</sub>/Hill slopes were: for BzATP at M283 vs T283 230 ± 22 μM/1.7 ± 0.13 vs 236 ± 25 μM/1.73 ± 0.1 and for ATP 920 ± 50 μM/1.9 ± 0.2 vs 870 ± 60 μM/1.8 ± 0.1. (d) Results from similar experiments performed at rat P2X<sub>7</sub>M283 and T283 (wildtype) receptor, points are from 4 – 6 experiments. EC<sub>50</sub> and Hill slopes were: for BzATP at M283 vs T283 6 ± 1 μM/1.6 ± 0.2 vs 8 ± 1 μM/1.8 ± 0.15 and for ATP 170 ± 25 μM/1.7 ± 0.14 vs 128 ± 30 μM/1.75 ± 0.3. There were no significant differences between M283 and T283 in agonist EC<sub>50</sub> values or Hill slopes.

**Figure 2** ATP-induced ethidium uptake is absent in cells expressing P2X<sub>7</sub>-M283. Time course of the ATP induced ethidium bromide uptake in HEK293 cells expressing mouse P2X<sub>7</sub>-T283 (circles) or M283 (squares) (a) or rat P2X<sub>7</sub>-T283 (circles) or M283 (squares) receptor (b). ATP (5 mM) was added when indicated

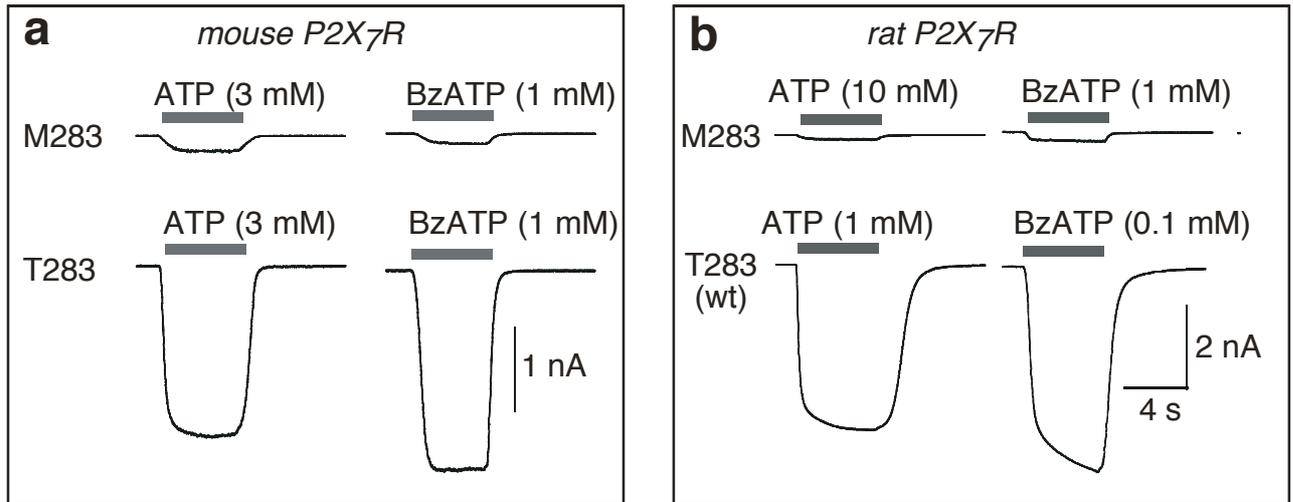
in low divalent external solution ( $n = 20$  cells in each case and is representative of four independent experiments). Digitonin ( $100 \mu\text{M}$ ) was added when indicated for the mouse or rat P2X<sub>7</sub>-M283 transfected cells to confirm ability of cells to take up dye.

**Figure 3** All mouse P2X<sub>7</sub> receptor mutations show equivalent expression at the cell surface. (a) Biotinylation experiments to determine the levels of surface protein for rat and mouse P2X<sub>7</sub>. Protein expression from transient transfections using three concentrations of cDNA (0.01, 0.1 and  $1 \mu\text{g}/\mu\text{l}$  as indicated) encoding rat P2X<sub>7</sub> wildtype (T283) receptor was compared to transfections with  $1 \mu\text{g}/\mu\text{l}$  mouse or rat P2X<sub>7</sub>-M283/P451, and mouse T283/P451 cDNA. Note that 10-fold lower concentration of rP2X<sub>7</sub> receptor cDNA ( $0.1 \mu\text{g}/\mu\text{l}$ ) than mouse P2X<sub>7</sub> receptor cDNA yielded equivalent total protein, surface expression and maximum current densities (a-c). Blots shown are representative results from experiments performed at least four times. (b) Current densities of rat and mouse P2X<sub>7</sub> constructs in response to maximum ATP application;  $n = 3-17$  for all experiments. (c) Relative surface expression of P2X<sub>7</sub> receptor protein expressed as a ratio of total P2X<sub>7</sub> receptor protein for all experiments in which  $\beta$ -actin levels were equivalent (as in a); no differences in total protein or surface expression of mouse P2X<sub>7</sub> receptors were observed.

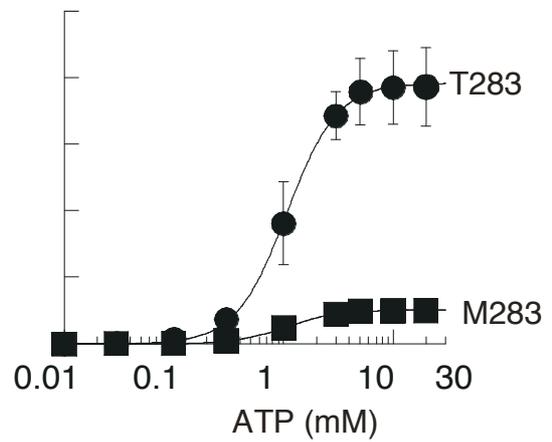
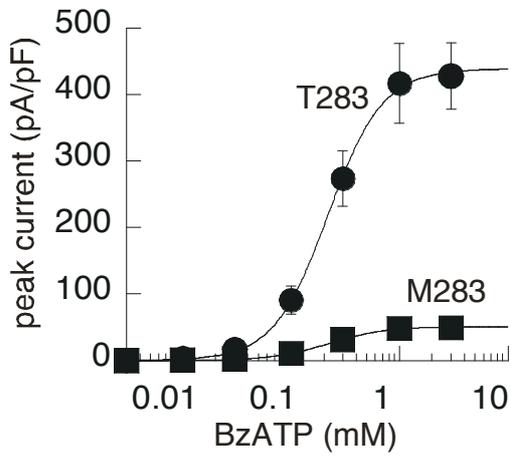
**Figure 4** The P451L polymorphism does not alter concentration-response curves for agonist-evoked membrane currents and does not significantly alter protein expression. (a,b) Representative currents in response to increasing concentrations of ATP (a) and BzATP (b) at mP2X<sub>7</sub>-T283/P451 and T283/L451 expressing cells; currents after response 'runup' had stabilized are shown. (c) Representative blot obtained as detailed in Figure 3 showing total and surface P2X<sub>7</sub> receptor protein with  $\beta$ -actin as the loading control. (d) Summary of all experiments as illustrated in (a,b);  $n = 3 - 12$  for each experiment.

**Figure 5** The P451L polymorphism inhibits ATP-induced ethidium uptake and rise in intracellular calcium concentration. (a) Time course ATP induced ethidium bromide uptake in cells expressing mouse P2X<sub>7</sub>-T283/P451 or T283/L451 as indicated. ATP (5 mM) was added at arrow in low divalent external solution. (b) ATP induced intracellular Ca<sup>2+</sup> changes in Fluo4 AM-loaded cells expressing same constructs as in (a). ATP (3mM) was applied for 60 s as indicated in low divalent external solution. Results in (a) and (b) are mean  $\pm$  s.e.m of 20 cells and are representative of four independent experiments in each case.

Figure 1



**c** *mouse P2X<sub>7</sub>R*



**d** *rat P2X<sub>7</sub>R*

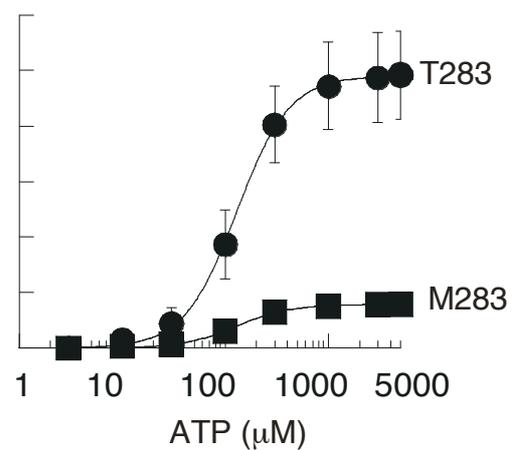
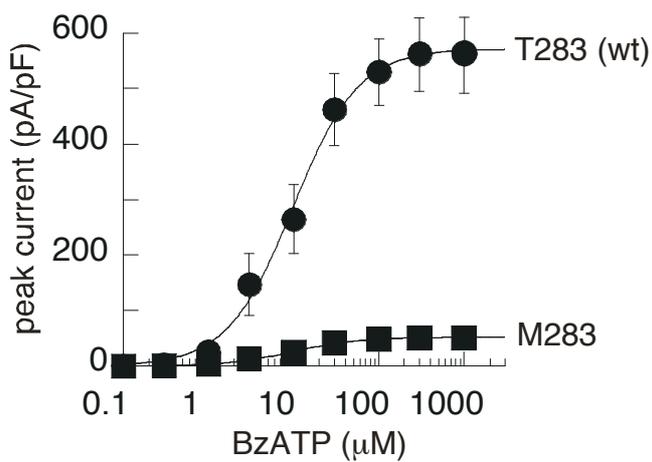
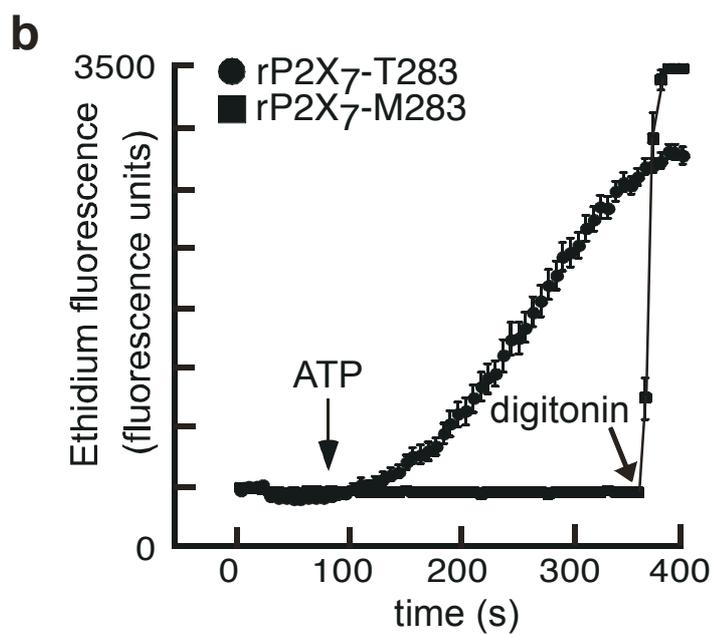
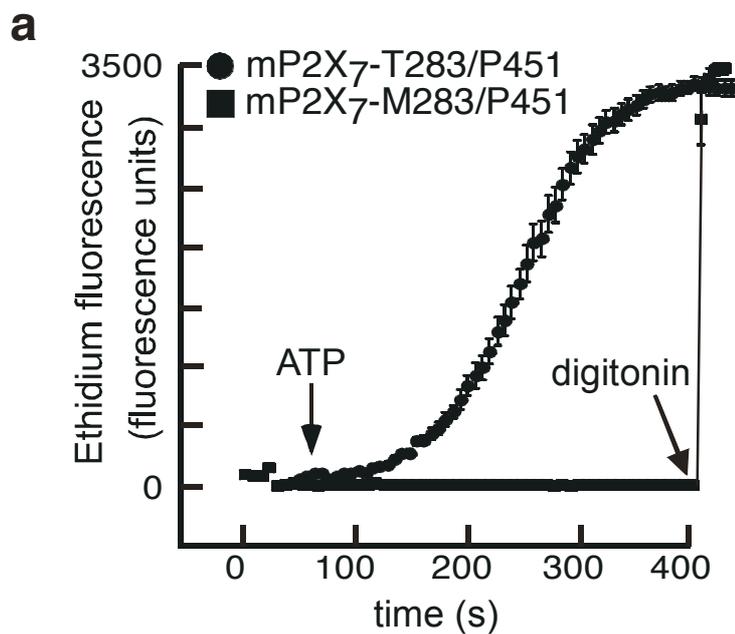
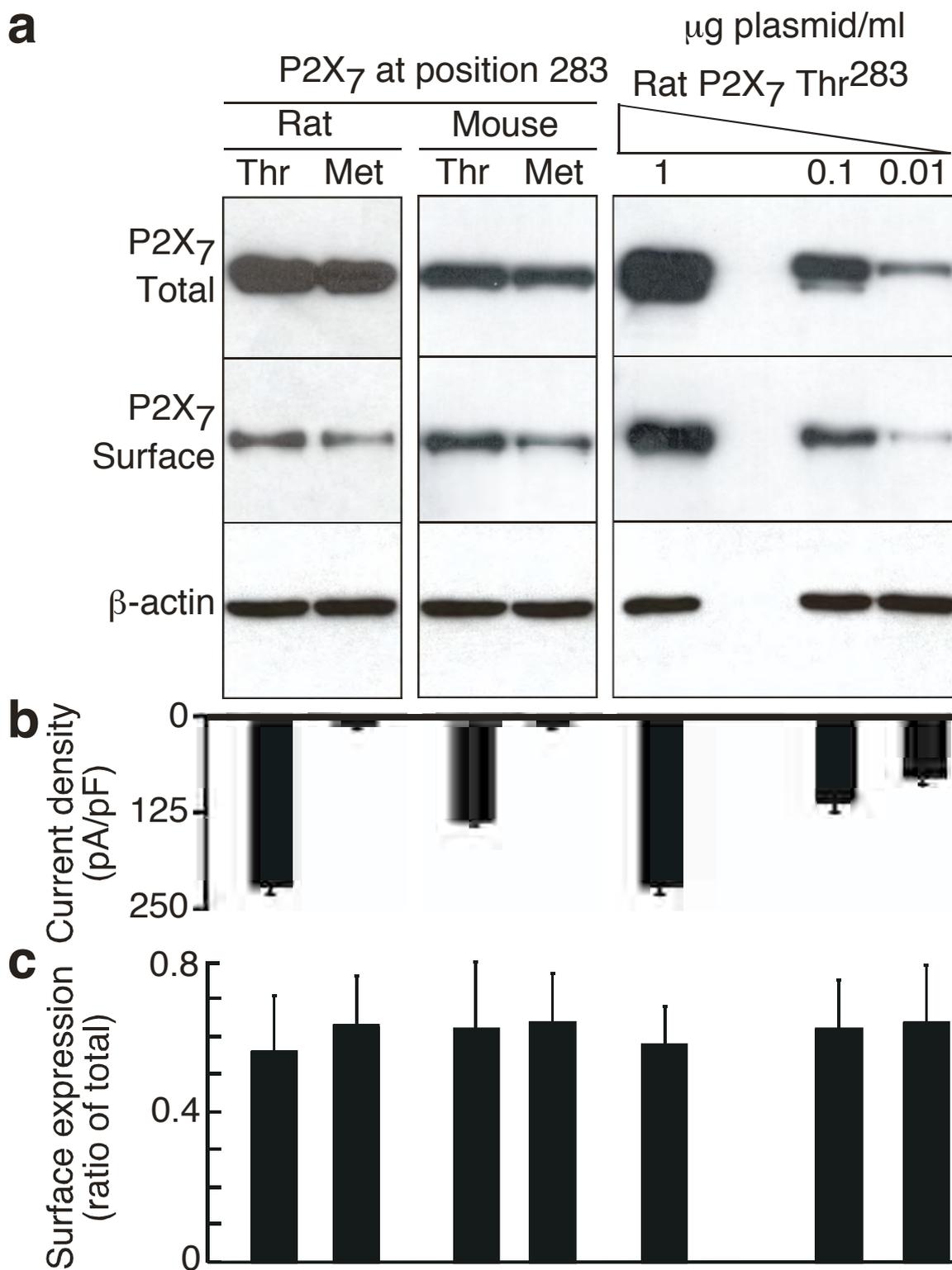


Figure 2





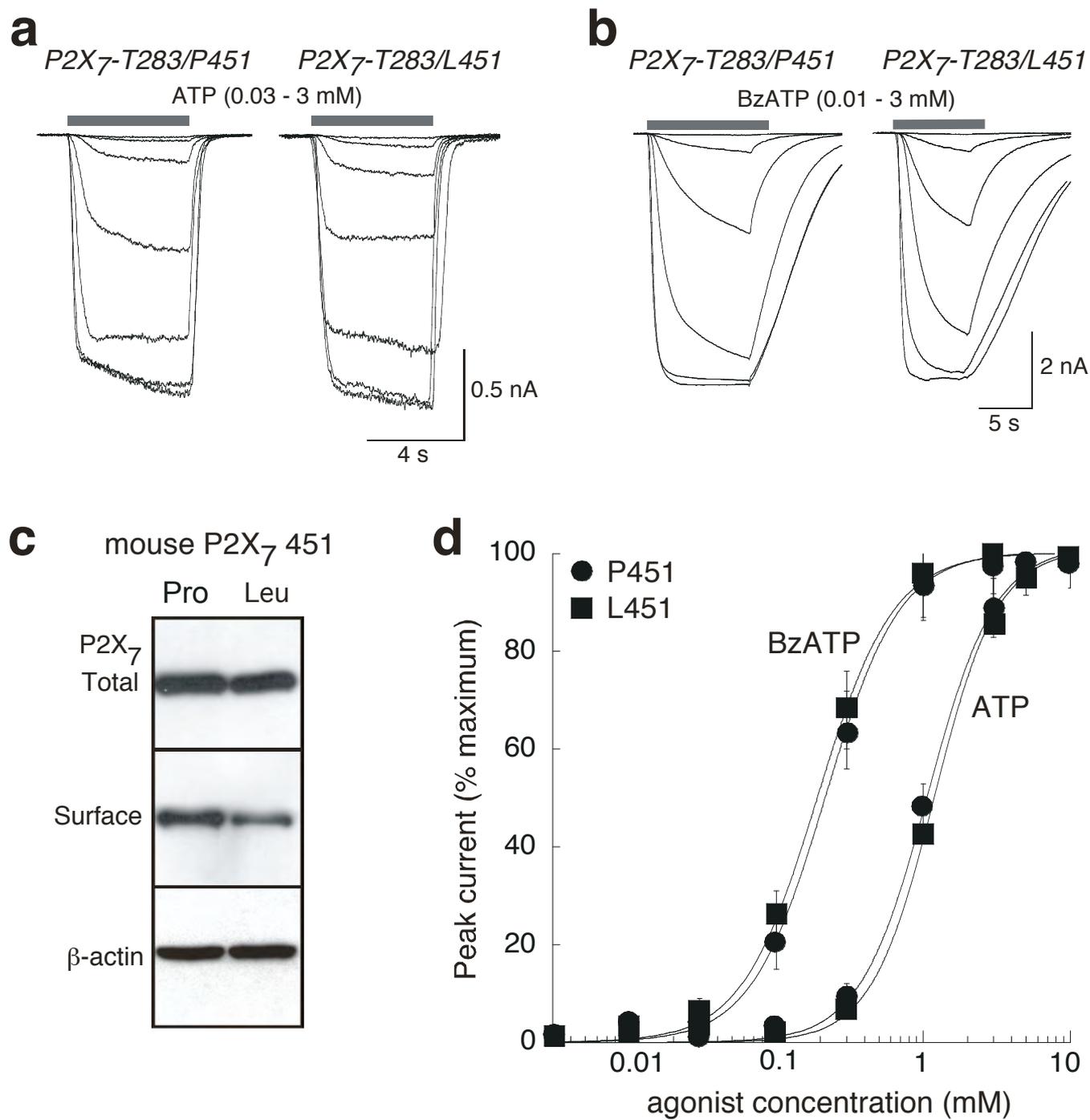


Figure 5

