R270C polymorphism leads to loss of function of the canine P2X7 receptor

Mari Spildrejorde
University of Wollongong, mgs840@uowmail.edu.au

Rachael Bartlett
University of Wollongong, rb401@uowmail.edu.au

Leanne Stokes
RMIT University

Iman Jalilian
University of Wollongong, ijhp032@uow.edu.au

Michelle Peranec
University of Wollongong, mp918@uowmail.edu.au

See next page for additional authors

Recommended Citation

R270C polymorphism leads to loss of function of the canine P2X7 receptor

Abstract

The relative function of the P2X7 receptor, an ATP-gated ion channel, varies between humans due to polymorphisms in the P2RX7 gene. This study aimed to assess the functional impact of P2X7 variation in a random sample of the canine population. Blood and genomic DNA were obtained from 69 dogs selected as representatives of a cross section of different breeds. P2X7 function was determined by flow cytometric measurements of dye uptake and patch-clamp measurements of inward currents. P2X7 expression was determined by immunoblotting and immunocytochemistry. Sequencing was used to identify P2RX7 gene polymorphisms. P2X7 was cloned from an English springer spaniel, and point mutations were introduced into this receptor by site-directed mutagenesis. The relative function of P2X7 on monocytes varied between individual dogs. The canine P2RX7 gene encoded four missense polymorphisms: F103L and P452S, found in heterozygous and homozygous dosage, and R270C and R365Q, found only in heterozygous dosage. Moreover, R270C and R365Q were associated with the cocker spaniel and Labrador retriever, respectively. F103L, R270C, and R365Q but not P452S corresponded to decreased P2X7 function in monocytes but did not explain the majority of differences in P2X7 function between dogs, indicating that other factors contribute to this variability. Heterologous expression of site-directed mutants of P2X7 in human embryonic kidney-293 cells indicated that the R270C mutant was nonfunctional, the F103L and R365Q mutants had partly reduced function, and the P452S mutant functioned normally. Taken together, these data highlight that a R270C polymorphism has major functional impact on canine P2X7.

Keywords

Purinergic receptor, missense mutation, single nucleotide polymorphism, monocyte, dog

Disciplines

Medicine and Health Sciences | Social and Behavioral Sciences

Publication Details

Authors

Mari Spildrejorde, Rachael Bartlett, Leanne Stokes, Iman Jalilian, Michelle Peranec, Vanessa Sluyter, Belinda Curtis, Kristen K. Skarratt, Amanda Skora, Tahani Bakhsh, Aine Seavers, Jason D. McArthur, Mark Dowton, and Ronald Sluyter

R270C polymorphism leads to loss of function of the canine P2X7 receptor

Mari Spildrejorde[1,2], Rachael Bartlett[1,2], Leanne Stokes[3,4], Iman Jalilian[1,2], Michelle Peranec[1,2], Vanessa Sluyter[1,2], Belinda L. Curtis[5], Kristen K. Skarratt[4], Amanda Skora[1,2], Tahani Bakhsh[1,2], Aine Seavers[6], Jason D. McArthur[1,2], Mark Dowton[1,2], Ronald Sluyter[1,2]

[1]School of Biological Sciences, University of Wollongong, Wollongong, Australia; [2]Illawarra Health and Medical Research Institute, Wollongong, Australia; [3]Health Innovations Research Institute, School of Medical Sciences, RMIT University, Bundoora, Australia; [4]Sydney Medical School Nepean, University of Sydney, Nepean Hospital, Penrith, Australia; [5]Albion Park Veterinary Hospital, Albion Park, Australia; [6]Oak Flats Veterinary Clinic, Oak Flats, Australia

Running head: A loss-of-function polymorphism of canine P2X7

Address for reprint requests and other correspondence: R. Sluyter, School of Biological Sciences, Illawarra Health and Medical Research Institute, University of Wollongong, Wollongong, NSW 2522, Australia. (email: rsluyter@uow.edu.au)

ABSTRACT

The relative function of the P2X7 receptor, an ATP-gated ion channel, varies between humans due to polymorphisms in the *P2RX7* gene. This study aimed to assess the functional impact of P2X7 variation in a random sample of the canine population. Blood and genomic DNA were obtained from 69 dogs selected as representatives of a cross-section of different breeds. P2X7 function was determined by flow cytometric measurements of dye uptake and patch-clamp measurements of inward currents. P2X7 expression was determined by immunoblotting and immunocytochemistry. Sequencing was used to identify *P2RX7* gene polymorphisms. P2X7 was cloned from an English Springer Spaniel and point mutations were introduced into this receptor by site-directed mutagenesis. The relative function of P2X7 on monocytes varied between individual dogs. The canine *P2RX7* gene encoded four missense polymorphisms: F103L and P452S, found in heterozygous and homozygous dosage; R270C and R365Q, found only in heterozygous dosage. Moreover, R270C and R365Q were associated with the Cocker Spaniel and Labrador Retriever, respectively. F103L, R270C and R365Q but not P452S corresponded to decreased P2X7 function in monocytes, but did not explain the majority of differences in P2X7 function between dogs, indicating that other factors contribute to this variability. Heterologous expression of site-directed mutants of P2X7 in human embryonic kidney-293 cells indicated that the R270C mutant was non-functional, the F103L and R365Q mutants had partly reduced function, and the P452S mutant functioned normally. Taken together, these data highlight that a R270C polymorphism has major functional impact on canine P2X7.

Keywords: purinergic receptor; missense mutation; single nucleotide polymorphism; monocyte; dog

INTRODUCTION

Since the partial (31) and complete (33) sequencing of the canine genome there has been considerable interest in the use of single nucleotide polymorphisms (SNPs) to help explain the ancestry and evolutionary origins of the domestic dog (*Canis lupus familiaris*) (33, 53), as well as the phenotypic diversity between breeds (9). Moreover, as exemplified by recent studies (30, 46), there is continual interest in identifying SNPs associated with breed-specific disorders to determine the causes of such disorders in dogs and to help elucidate the mechanisms involved in similar disorders within humans. One study that supports this approach has been a genome-wide association analysis, which identified a SNP within the gene coding canine Cu/Zn SOD in breeds susceptible to degenerative myelopathy (3); a disease similar to amyotrophic lateral sclerosis in humans, which is also associated with SNPs in the gene coding human Cu/Zn SOD (43). This approach however is currently restricted. First, presumably not all SNPs have been identified in the some 350 breeds that exist. Second, as for humans the molecular and cellular effects of most canine SNPs identified to date are yet to be fully elucidated. Thus, there is a need to identify novel SNPs within the dog, and to define how these and existing SNPs influence canine biology.

The P2X7 receptor is a trimeric ligand-gated ion channel present on various cell types, with high amounts present on the cell-surface of monocytes (54). Upon activation by its natural ligand ATP, the P2X7 channel rapidly opens and dilates to form a pore to allow the flux of organic cations and anions including fluorescent dyes (11). P2X7 activation also results in a variety of downstream events including the release of pro-inflammatory mediators such as IL-1β, PGE$_2$ and CXC chemokine ligand 16 (4, 38, 40). Conversely in the absence of ATP, P2X7 may function as a scavenger receptor to facilitate the phagocytosis of bacteria and apoptotic cells (23). As a result of these and other observations, P2X7 is attracting

considerable interest as a therapeutic target in various immune-mediated disorders and pain (36). P2X7 activation is also important in physiological systems other than the immune system such as bone homeostasis (52), and carbohydrate and lipid metabolism (2, 6).

The human, canine, rhesus macaque, rat and murine P2X7 subunits have been cloned (10, 12, 41, 42, 51). Each of these mammalian P2X7 subunits are 595 amino acids in length, and comprise intracellular N- and C-termini, two trans-membrane domains and a large extracellular loop. The P2X7 subunit is encoded by the *P2RX7* gene, which comprises 13 exons and is highly polymorphic in humans, resulting in at least 12 loss-of-function and three gain-of-function receptor variants (45). A loss-of-function SNP, P451L (rs48804829), has also been described in mouse P2X7 (1), but descriptions of SNPs in the *P2RX7* gene from other species are limited.

Some 40 years ago, Parker and Snow demonstrated that extracellular ATP could increase the membrane permeability of Beagle erythrocytes to cations (37). Nearly 30 years later, our group using erythrocytes from English Springer Spaniels, identified that this effect was mediated by P2X7 (44), providing the first direct evidence that functional P2X7 receptors are present within dogs. Subsequently our group demonstrated that P2X7 was also present on monocytes, B cells and T cells from this (44, 48), and more recently from other dog breeds (25, 47). However, the dog breeds in which P2X7 has been studied are limited, and thus it remains unknown if P2X7 function varies between dogs or breeds. Two missense (non-synonymous) SNPs, F103L (rs23314713) and P452S (rs23315462), as well as one synonymous SNP N131N (rs8660017) in the canine *P2RX7* gene have been reported in databases of Ensembl (http://www.ensembl.org/index.html) or the National Centre for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/snp). However, the functional effects of these SNPs remain unknown. The current study describes that the relative P2X7 function varies between dogs. Moreover, the current study confirms the

presence of the F103L and P452S SNPs in canine P2X7, and identifies three other missense SNPs, R270C, R365Q and L440F. Finally, using a recombinant P2X7, cloned from an English Springer Spaniel, the current study demonstrates that the R270C SNP results in near complete loss of function of canine P2X7.

MATERIALS AND METHODS

Materials. Ficoll-Paque PLUS was from GE Healthcare Biosciences (Uppsala, Sweden). YO-PRO-1 iodide solution, ExoSAP-IT, DMEM/F12 medium, penicillin-streptomycin, OPTI-MEM Reduced Serum Medium and Lipofectamine 2000 were from Invitrogen (Grand Island, NJ). ATP, 2'(3')-O-(4-benzoyl)benzoyl ATP (BzATP), adenosine 5'-O-(3-thio)triphosphate (ATPγS), ADP, UTP, αβ-methylene ATP (αβ-meATP), Brilliant Blue G (BBG) and propidium iodide were from Sigma Chemical Co (St Louis, MO). Allophycocyanin (APC)-conjugated anti-canine CD14 MAb (clone M5E2) was from BioLegend (San Diego, CA). SPHERO Rainbow Fluorescent Particles were from Spherotech (Lake Forest, IL). Primers for sequencing were obtained from GeneWorks (Hindmarsh, Australia). MangoTaq DNA polymerase was from Bioline (London, UK). Primers for cloning were from Sigma Life Science (Castle Hill, Australia). PfuUltra High-Fidelity DNA polymerase was from Stratagene (La Jolla, CA). *Nhe*I and *Not*I restriction enzymes were from Fermentas (Glen Burnie, MD). FBS was from Bovogen Biologicals (East Keilor, Australia). Ethidium bromide was from Amresco (Solon, OH). Rabbit anti-mouse P2X7 (extracellular epitope) polyclonal antibody (Ab) and blocking peptide, and rabbit anti-rat P2X7 (C-terminal epitope) Ab were from Alomone Labs (Jerusalem, Israel). A438079, AZ10606120 and AZ11645373 were from Tocris Bioscience (Ellisville, MO). KN-62 was from Alexis Biochemicals (Lausen, Switzerland).

Samples. All canine samples were collected from privately owned dogs presented at local veterinary clinics with informed consent of owners. All samples were collected in accordance with institutional guidelines, and with approval from the Animal and Human Ethics Committees of the University of Wollongong (Wollongong, Australia).

YO-PRO-1^{2+} uptake into canine monocytes. Peripheral blood was collected into VACUETTE lithium heparin tubes (Greiner Bio-One, Frickenheisen, Germany) from either pedigree or cross breed dogs. Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats using Ficoll-Paque density centrifugation as described (48), washed twice with phosphate-buffered saline and once with NaCl medium (145 mM NaCl, 5 mM KCl, 5 mM glucose, 0.1% BSA, 10 mM HEPES, pH 7.4). PBMCs in NaCl medium (2 x 10^6 cells/mL) were pre-incubated for 5 min at 37°C before addition of 1 μM YO-PRO-1^{2+} followed 15 sec later by the addition of 1 mM ATP. PBMCs were incubated in the absence or presence of ATP for 5 min, before the addition of an equal volume of ice-cold NaCl medium containing 20 mM MgCl$_2$ (MgCl$_2$ medium) and centrifugation. PBMCs were washed once with NaCl medium and then labelled with APC-conjugated anti-CD14 mAb. Data were acquired using a BD (San Jose, CA) LSR II flow cytometer, with a total of 1 x 10^5 events collected using emission windows of 515/20 and 660/20 nm for YO-PRO-1^{2+} and APC, respectively. For each sample, the detector for 515/20 nm was adjusted using SPHERO Rainbow Fluorescent Particles to a mean fluorescence of 10,000 using BD FACSDiva software. The mean fluorescence intensity (MFI) of YO-PRO-1^{2+} uptake was determined using FlowJo software (Tree Star, Ashland, OR). ATP-induced YO-PRO-1^{2+} uptake is presented as the mean difference (of duplicate samples) between YO-PRO-1^{2+} uptake in the presence and absence of ATP.

P2RX7 gene sequencing. DNA was isolated from canine peripheral blood (collected as above) using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI) according to the manufacturer's instructions and stored at -80°C. Twelve primer pairs for exons 1-12 and

two overlapping primer pairs for exon 13 (Table 1) were used to amplify individual exons of the canine *P2RX7* gene by PCR. The PCR was conducted using MangoTaq DNA polymerase with an initial denaturation at 94°C for 1 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 58.6-64.5°C (Table 1) for 30 sec, and extension at 72°C for 1 min. After the cycling was completed, a final extension at 72°C for 5 min was performed, and the reactions returned to room temperature. The sequences of ExoSAP-IT treated amplicons were determined using the above primers with the ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA) and an Applied Biosystems 3130xl Genetic Analyzer. Resulting sequences were compared to the NCBI Reference Sequence NM_001113456.1, which was defined as wild-type canine P2X7.

Cloning of canine P2X7. cDNA from PBMCs of an English Springer Spaniel was kindly provided by Peter Williamson and Rosanne Taylor (University of Sydney, Sydney, Australia) and used as a template to clone canine P2X7 using standard techniques. Canine P2X7 cDNA was amplified by PCR using PfuUltra High-Fidelity DNA polymerase and the product was cloned into the *Nhe*I/*Not*I sites of a pHumanP2X7-AcGFP-N1 plasmid (49), from which human P2X7-AcGFP had been removed by double digestion with *Nhe*I/*Not*I. The sequence of the resulting plasmid containing canine P2X7, pEnglish Springer Spaniel P2X7, was determined using four overlapping forward and reverse primer pairs (5' to 3': CCGCCTGGTGTCCCATCG and ATCTTCTTGATTCCATTCTCC; CCACCTCAGAGCCGTTCA and CAGCTGCATCTCCTCTG; TGGCACCATTAAGTGGAT and GGTTCTGAGTCTTGTGAAAGGT; AGACAATTGTGGAGCCAA and CTAGGTCCTGGGAGCCAAAG) with the ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit and an Applied Biosystems 3130xl Genetic Analyzer. Resulting sequences were compared to the NCBI Reference Sequence NM_001113456.1, the earlier NCBI Reference Sequence XM_534669.2 (originally obtain from a Boxer as part of the canine genome project) (33) and to the sequence of P2X7 previously cloned from heart cDNA of a non-disclosed dog breed

(42). Plasmid DNA was isolated using the Wizard Plus SV Minipreps DNA Purification System (Promega) according to the manufacturer's instructions and stored at -20°C.

Cell lines. Human embryonic kidney (HEK)-293 and murine microglial EOC13 cells were from the American Type Culture Collection (Rockville, MD). Madin-Darby canine kidney (MDCK) cells were from the European Collection of Cell Cultures (Porton Down, UK). HEK-293 and MDCK cells were maintained in DMEM/F12 medium supplemented with 10% FBS, 2 mM GlutaMAX, 100 U/mL penicillin and 10 μg/mL streptomycin (complete culture medium) at 37°C/5% CO_2. Murine microglial EOC13 cells were maintained as described (5).

Cell transfections. HEK-293 cells in complete culture medium (0.25-0.5 x 10^6 cells/mL) were incubated overnight at 37°C/5% CO_2. Cells were then incubated in complete culture medium containing 4 μg plasmid DNA and Lipofectamine 2000 (diluted in OPTI-MEM Reduced Serum Medium) for 24 h at 37°C/5% CO_2. The medium was then replaced and the cells incubated for a further 24 h at 37°C/5% CO_2.

Fluorescent dye uptake into HEK-293 and MDCK cells. The uptake of ethidium$^+$ (or YO-PRO-1^{2+} or propidium^{2+} where indicated) into HEK-293 and MDCK cells in NaCl medium was determined by flow cytometry as described (18).

Immunoblotting. Whole lysates of HEK-293 or EOC13 cells were prepared as described (13). Equal amounts of total protein were separated under reducing conditions using Any kD Mini-PROTEAN TGX Stain-Free Gels and transferred to nitrocellulose membrane using a Trans-Blot Turbo Transfer System (all Bio-Rad, Hercules, CA). Immunoblotting was performed with an anti-P2X7 polyclonal Ab as described (13). To confirm equal protein loading within stain-free gels, total protein was measured using a Bio-Rad Criterion Stain Free Imager and Image Lab software. P2X7 (75 kDa) was detected using Amersham Hyperfilm ECL (GE Healthcare, Little Chalfont, UK), and relative P2X7 expression was measured using a Bio-Rad GS-800 Densitometer and ImageJ software (http://rsbweb.nih.gov/ij/).

Site-directed mutagenesis. Missense SNPs were introduced into pEnglish Springer Spaniel P2X7 using standard techniques and confirmed by sequencing (SUPAMAC, Sydney, Australia) as described previously (49).

Immunocytochemistry. Immunolabeling of non-permeabilized cells was performed with an anti-mouse P2X7 polyclonal Ab as described (5). Cells were visualized using a TCS SP5 II confocal imagining system (Leica, Mannheim, Germany) (excitation at 561 nm; emission collected at 575-630 nm).

Electrophysiology. Whole cell patch-clamp recordings of HEK-293 cells were performed in low divalent solution (145 mM NaCl, 5 mM KCl, 0.2 mM $CaCl_2$, 13 mM glucose, 10 mM HEPES, pH 7.3, osm 295-310) and recorded using a HEKA (Lambrecht, Germany) EPC10 amplifier as described (7).

Data presentation and statistical analyses. Errors are expressed as SDs. Differences between groups were compared using the One-way ANOVA (using Tukey's multiple comparison test). Measurements of ATP-induced YO-PRO-1^{2+} uptake in canine monocytes reflected a non-Gaussian distribution (as determined by the D'Agostino & Pearson omnibus normality test), thus differences in ATP-induced YO-PRO-1^{2+} uptake between *P2RX7* genotypes were compared using the Mann-Whitney test or the Kruskal-Wallis test (using Dunn's multiple comparison test). Statistical comparisons were made using Prism 5 (GraphPad Software, San Diego, CA) with $P<0.05$ considered significant.

RESULTS

To determine if the relative P2X7 function varied between dogs, blood was obtained from dogs presented at local veterinary clinics and the ATP-induced uptake of YO-PRO-1^{2+} into CD14$^+$ monocytes determined by flow cytometry. Calibration beads were used to set the

515/20 nm detector of the flow cytometer for each sample. ATP induced YO-PRO-1^{2+} uptake into monocytes from all 52 dogs studied, with an average MFI of 152 ± 97 (Fig. 1). Relative P2X7 function varied between dogs. Within individual breeds where three or more dogs were studied, relative P2X7 function varied by at least two-fold in both American Bull Terriers and Staffordshire Bull Terriers, and up to 13-fold in Labrador Retrievers (Fig. 1). Similarly, relative P2X7 varied amongst cross breed dogs with a 13-fold difference in P2X7 function between monocytes from a Border Collie and Australian Cattle Dog cross, and from a Golden Retriever and Labrador Retriever cross, which had the highest (MFI of 395) and lowest (MFI of 31) P2X7 function, respectively (Fig. 1). In contrast to canine monocytes, ATP was unable to induce YO-PRO-1^{2+} uptake in MDCK cells over 5 min (MFI of 0; Fig. 1).

To determine if the variation in P2X7 function between dogs was attributed to SNPs in the *P2RX7* gene, the 13 exons of this gene from 19 dogs with either low or high P2X7 function, as well as from MDCK cells were sequenced. Sequences were compared to the NCBI Reference Sequence NM_001113456.1, which was designated as the wild-type canine P2X7 sequence. Sequencing confirmed the existence of the two previously reported missense SNPs, rs23314713 (ttc>ctc) in exon 3 and rs23315462 (cct>tct) in exon 13, which code for the amino acid substitutions F103L and P452S, respectively (Fig. 2*A*; Table 2). Sequencing also confirmed the existence of the previously reported synonymous SNP rs8660017 (aat>aac; N131N) in exon 4 of 14 dogs in either heterozygous (*n*=6) or homozygous dosage (*n*=8) (*results not shown*). Finally, sequencing identified two novel missense SNPs, cgt>tgt in exon 8 and cga>caa in exon 11, which code for the amino acid substitutions R270C and R356Q, respectively (Fig. 2*A*; Table 2). Complete sequences were obtained from all exons except for exon 13, for which the 48 base pairs coding the last 16 amino acid residues of the C-terminus of P2X7 could not be resolved due to poor signal-to-noise ratio (*results not shown*).

Next, exons 3, 8, 11 and 13 of the *P2RX7* gene were sequenced from the remaining dogs with P2X7 functional data, as well as from 17 other dogs for which genomic DNA was available. The allele frequencies of F103L, R270C, R365Q and P452S were 0.38, 0.01, 0.03 and 0.39, respectively (Table 2). Notably, the R270C SNP was only observed in a Cavalier King Charles Spaniel and Cocker Spaniel cross and in MDCK cells, which were originally derived from a Cocker Spaniel (16). In both instances the R270C SNP was present in heterozygous dosage, and appeared to be linked with the P452S SNP (Table 2). The R270C SNP was not observed in a second Cocker Spaniel cross, from which no functional data was available (Table 2). The R365Q SNP was only observed in three Labrador Retrievers and in a Golden Retriever and Labrador Retriever cross, and then only in heterozygous dosage in all four cases; three other Labrador Retrievers were wild-type at residue position 365 (Table 2).

The relative P2X7 function obtained above (Fig. 1) was then compared to each missense SNP individually. The P2X7 function of dogs heterozygous for the F103L SNP was similar to that of dogs wild-type for F103, however the P2X7 function of dogs homozygous for this SNP was significantly lower compared to wild-type animals (Fig. 2*B*). Sample numbers were limited for both the R270C and R365Q SNPs, and were only observed in heterozygous dosage, but the mean P2X7 function in dogs with either of these SNPs was about half that of the mean P2X7 function in dogs wild-type for R270 or R365 (Fig. 2*B*). In contrast, mean P2X7 function was similar between dogs wild-type for P452, or heterozygous or homozygous for the P452S SNP (Fig. 2*B*).

Our group has previously characterized P2X7 in erythrocytes and leukocytes from English Springer Spaniels (44, 48). Therefore, P2X7 was cloned using cDNA from this breed to generate a recombinant P2X7 for site-directed mutagenesis studies. The cloned receptor was 595 amino acids in length (*results not shown*) and was wild-type at amino acid positions 103 (F103), 270 (R270) and 365 (R365) (Fig. 3*A*) compared to the (wild-type) NCBI Reference

Sequence NM_001113456.1. In contrast, the cloned receptor differed at amino acid positions 440 (L440F; ttg>ttt) and 452 (P452S; cct>tct) (Fig. 3*A*).

To determine if the P2X7 cloned from an English Springer Spaniel could be expressed heterologously as a recombinant receptor in mammalian cells, whole lysates of HEK-293 cells transfected with English Springer Spaniel P2X7 plasmid DNA or mock-transfected HEK-293 cells were examined by immunoblotting using an Ab against an extracellular or C-terminal epitope of P2X7. Murine EOC13 cells, which express endogenous P2X7 (5), were used as a positive control. Both antibodies detected a major band at 75 kDa, the predicted size of glycosylated P2X7, in HEK-293 cells expressing English Springer Spaniel P2X7 and EOC13 cells, but not mock-transfected HEK-293 cells (Fig. 3*B*).

Next, to determine if the P2X7 cloned from an English Springer Spaniel could form functional receptors when expressed heterologously in mammalian cells, a flow cytometric assay was used to measure ATP-induced fluorescent cation uptake into HEK-293 cells expressing English Springer Spaniel P2X7 or mock-transfected HEK-293 cells. ATP failed to induce ethidium$^+$ uptake into mock-transfected HEK-293 cells (Fig. 3*C*). In contrast, ATP induced robust ethidium$^+$ uptake into HEK-293 cells expressing English Springer Spaniel P2X7 compared to corresponding cells incubated in the absence of ATP (Fig. 3*C*). ATP also induced robust uptake of two other fluorescent cations, YO-PRO-1^{2+} (375 Da) and propidium^{2+} (415 Da), as well ethidium$^+$ (314 Da), into HEK-293 cells expressing English Springer Spaniel P2X7 (Fig. 3*D*).

The recombinant English Springer Spaniel P2X7 expressed heterologously in HEK-293 cells was further characterized using flow cytometric measurements of ethidium$^+$ uptake. ATP induced ethidium$^+$ uptake in a concentration-dependent fashion with an EC$_{50}$ value of 253 μM (Fig. 3*E*). Moreover, BzATP and ATPγS, agonists of endogenous and recombinant human P2X7 (15, 19), induced ethidium$^+$ uptake in a concentration-dependent fashion and with EC$_{50}$ values

of 13 and 438 μM, respectively (Fig. 3*E*). Maximum ethidium$^+$ uptake was observed with 50 μM BzATP, while ATP and ATPγS represented partial agonists. In contrast, ADP, UTP and αβ-meATP (each at 1 mM) failed to induce ethidium$^+$ uptake (Fig. 3*E*).

The efficacy of five well-known human and rodent P2X7 antagonists was then tested against the recombinant English Springer Spaniel P2X7. A438079 (35), AZ10606120 (34), AZ11645373 (50), BBG (28) and KN-62 (20) impaired ATP-induced ethidium$^+$ uptake in a concentration-dependent fashion with IC$_{50}$ values of 195 nM, 11 nM, 9 nM, 1000 nM and 16 nM, respectively (Fig. 3*F*). Moreover, all five antagonists completely impaired ATP-induced ethidium$^+$ uptake with maximal inhibition observed at concentrations of 0.1 μM for AZ10606120 and AZ11645373, 1 μM for KN-62, and 10 μM for A438079 and BBG (Fig. 3*F*).

To determine if any of the missense SNPs identified above (F103L, R270C, R365Q, L440F and P452S) effect P2X7 function, the cloned English Springer Spaniel P2X7 was mutated as follows. First, the plasmid containing the cloned English Springer Spaniel P2X7 (which contained both the 440F and 452S SNPs; now termed 440F/452S mutant P2X7) was changed to wild-type at position 440 (termed 452S mutant P2X7) by mutating the phenylalanine residue to leucine. Next, the plasmid containing only the 452S SNP was either changed to wild-type P2X7 (termed wild-type P2X7) by mutating the serine residue at position 452 to proline or changed to a double mutant (termed 270C/452S mutant P2X7) by mutating the arginine residue at position 270 to cysteine. Finally, the wild-type P2X7 plasmid was mutated to contain either the 103L or 365Q SNP (termed 103L and 365Q mutant P2X7, respectively).

Using the above plasmids, the effect of each SNP on P2X7 function assessed by measurements of ATP-induced ethidium$^+$ uptake into transfected HEK-293 cells. ATP-induced ethidium$^+$ uptake was 24% less in cells expressing 452S mutant P2X7 compared to cells expressing 440F/452S mutant P2X7 (Fig. 4*A*). ATP-induced ethidium$^+$ uptake into cells

expressing 452S mutant P2X7 was similar to that of cells expressing wild-type P2X7 (Fig. 4*B*). In contrast, ATP-induced ethidium$^+$ uptake was almost completely abolished in cells expressing 270C/452S mutant P2X7 compared to cells expressing either wild-type or 452S mutant P2X7 (Fig. 4*B*). Finally, ATP-induced ethidium$^+$ uptake was 46% and 37% less in cells expressing either 103L or 365Q mutant P2X7, respectively compared to cells expressing wild-type P2X7 (Fig. 4*C*).

To determine if the differences in ATP-induced ethidium$^+$ uptake in transfected HEK-293 cells related to total P2X7 expression, whole lysates of HEK-293 cells transfected with DNA from wild-type or each mutant P2X7 plasmid were examined by immunoblotting using the Ab against the extracellular epitope of P2X7 and the amount of P2X7 quantified. As above (Fig. 3*B*), a major band at 75 kDa was detected in lysates of HEK-293 cells expressing wild-type or mutant P2X7 (Fig. 4*D-F*). The amount of P2X7 was 29% less in cells expressing the 452S mutant compared to cells expressing 440F/452S mutant P2X7 (Fig. 4*D*). The amount of P2X7 was 22% and 30% less in cells expressing either 452S or 270C/452S mutant P2X7 respectively compared to cells expressing wild-type P2X7 (Fig. 4*E*). The amount of P2X7 was 48% and 32% less in cells expressing either 103L or 365Q mutant P2X7 respectively compared to cells expressing wild-type P2X7 (Fig. 4*F*).

The above data indicates that the R270C SNP has a major functional impact on canine P2X7. Therefore, this SNP was characterised further in HEK-293 cells expressing either wild-type, 452S or 270C/452S mutant P2X7. First, patch-clamp measurements revealed that ATP induced robust inward currents in HEK-293 cells expressing wild-type or 452S mutant P2X7, with mean current densities of 49 (19) pA/pF (*n*=6) and 39 (24) pA/pF (*n*=7), respectively and which were not significantly different (*P*>0.05) (Fig. 5*A*). In contrast, ATP-induced inward currents in HEK-293 cells expressing 270C/452S mutant P2X7 were significantly smaller (mean current density of 3 (1) pA/pF, *n*=5) than those observed in HEK-293 cells expressing

wild-type or 452S mutant P2X7 ($P<0.01$ and $P<0.05$, respectively) (Fig. 5*A*). In addition, multiple applications of ATP saw wild-type or 452S mutant P2X7 display facilitation of responses over 5 min, whereas 270C/452S mutant P2X7 responses did not display facilitation and remained small (Fig. 5*B*). Second, immunolabeling with an Ab specific for the extracellular loop of P2X7 and confocal microscopy demonstrated cell-surface expression of P2X7 on HEK-293 cells expressing wild-type, 452S or 270C/452S mutant P2X7 (Fig. 6). In contrast, immunoreactivity of mock-transfected HEK-293 cells with anti-P2X7 Ab, or HEK-293 cells expressing wild-type or mutant P2X7 incubated in the presence of anti-P2X7 Ab with blocking peptide was minimal (*results not shown*).

DISCUSSION

The current study aimed to assess the functional impact of P2X7 variation in a random sample of the canine population. Others and us have previously shown that monocyte P2X7 function correlates with *P2RX7* genotype in humans (14, 24, 49). Thus, the variable P2X7 function between dog breeds and within individual breeds may in part be caused by SNPs within the canine *P2RX7* gene. Of the *P2RX7* SNPs identified F103L, R270C and R365Q, but not P452S, corresponded to decreased P2X7 function in canine monocytes. However these SNPs could not explain the majority of differences in P2X7 function between dogs, indicating that other factors contribute to this variability. Such factors may include SNPs in the last 48 base pairs of exon 13 and in the promoter and intronic regions of the *P2RX7* gene, differential expression of P2X7 splice variants, or differences in the health status of dogs upon presentation at local veterinary clinics.

Of the SNPs identified in our dog population and our cloned English Springer Spaniel P2X7 (Fig. 7), the R270C SNP was concluded to be a loss-of-function SNP impairing both P2X7

channel activity and pore formation. To the best of our knowledge this SNP has not been previously reported, but a R270H SNP (rs7958311) has been identified in human P2X7 and confers a partial loss of function (49). Thus, this latter result indirectly supports the concept that R270C, which exchanges a positively charged arginine residue for a polar, uncharged cysteine residue, is a loss-of-function SNP in canine P2X7. Structural modeling of human P2X7 places the R270 residue and the neighboring R276 residue, which is the site for the R276H loss-of-function SNP (49), in the β12 strand of the lower body of the extracellular loop and away from the ATP-binding site (27). As such, Jiang and colleagues proposed that the R276H SNP maybe involved in conformational changes associated with channel gating or receptor activation/deactivation (27). Thus by extension, it is possible that the R270C SNP may also act in a similar way to negatively effect P2X7 function. Alternatively, since this SNP introduces a cysteine adjacent to the existing cysteine at position 269, it is possible that the R270C SNP disrupts the potential disulfide bond between C260 and C269 (29) to reduce receptor function by altering the structure of the P2X7 subunit. Disulfide bonds of the extracellular loop of P2X7 are also essential for its trafficking to the plasma membrane (29), however it appears unlikely that R270C SNP impairs trafficking of the canine P2X7 to the cell-surface as immunocytochemistry showed the presence of cell-surface P2X7 on HEK-293 cells expressing P2X7 containing this SNP.

The missense SNPs, F103L and R365Q, in canine P2X7 may be partial loss-of-function SNPs. However the current body of evidence is limited as total P2X7 expression was also reduced by a similar amount in HEK-293 cells transfected with either mutant P2X7 compared to cells transfected with wild-type P2X7. Thus, whether the lower P2X7 function of either mutant receptor is due to reduced P2X7 protein synthesis or half-life, or plasmid transfection efficiency remains to be determined.

In contrast to the other missense SNPs in canine P2X7, it is reasonable to conclude that the P452S SNP does not alter P2X7 function. It is noteworthy that the P452S SNP in canine P2X7 is located at the equivalent position to that of the partial loss-of-function P451L SNP in murine P2X7, which is present in some but not all mouse strains (1). This difference in effect between canine P452S and murine P451L on P2X7 function may relate to differences between the hydrophilic and larger hydrophobic side chain of serine and leucine, respectively.

The current study involved the cloning of canine P2X7 from an English Springer Spaniel for use in site-directed mutagenesis studies to examine the effects of identified SNPs on P2X7 function. This breed was originally chosen following our original studies of endogenous P2X7 in this breed (44, 48). Compared to wild-type canine P2X7 (NCBI Reference Sequence NM_001113456.1), the cloned English Springer Spaniel P2X7 differed by two amino acid residues, L440F and P452S. The P452S SNP had an allele frequency of 0.39 in our dog population. In contrast, the L440F SNP has not been previously reported and was not observed in any other dog for which exon 13 was sequenced (*results not shown*). Whether this variant represents a SNP in English Springer Spaniels or was introduced as a result of the cloning process remains unknown.

Pharmacological characterization of the recombinant English Springer Spaniel P2X7 demonstrated that this receptor displays rank orders of agonist and antagonist potencies similar to that of endogenous and recombinant human P2X7 (15, 19, 39). Of note, the EC_{50} values for BzATP and ATP were similar to that described for endogenous P2X7 in erythrocytes from English Springer Spaniels (48). Moreover, study of both endogenous P2X7 (48) and the recombinant English Springer Spaniel P2X7 revealed that ATP was a partial agonist compared to BzATP. These results contrast findings with the previously cloned canine P2X7 of Roman and colleagues, which demonstrated that BzATP was a partial agonist of canine P2X7 compared to ATP (42). The reason for this difference between these studies

remains unknown but one possible explanation is the amino acid at position 103 alters BzATP sensitivity.

In conclusion, the current study identified a missense SNP, R270C, which leads to a loss of function of canine P2X7. It will be of importance in future studies to determine the frequency of this SNP in Cocker Spaniels and other breeds, and to determine whether this SNP disrupts P2X7-mediated physiological processes, such as ATP-induced IL-1β release. Moreover, since SNPs in the human *P2RX7* gene are associated with various diseases (17, 21, 22, 32), and that a three base-pair deletion in the gene coding the P2Y12 receptor is associated with post-operative hemorrhage in a Greater Swiss Mountain dog (8), it will be of interest to determine whether the R270C SNP or other SNPs in the canine *P2RX7* gene are associated with disease in dogs.

and the National Health and Research Council of Australia (Project Grant 632687). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

DISCLOSURES

No conflict of interest, financial or otherwise, are declared by the author(s).

REFERENCES

1. **Adriouch S, Dox C, Welge V, Seman M, Koch-Nolte F, Haag F**. Cutting edge: a natural P451L mutation in the cytoplasmic domain impairs the function of the mouse P2X7 receptor. *J Immunol* 169: 4108-4112, 2002.
2. **Amoroso F, Falzoni S, Adinolfi E, Ferrari D, Di Virgilio F**. The P2X7 receptor is a key modulator of aerobic glycolysis. *Cell Death Dis* 3: e370, 2012.
3. **Awano T, Johnson GS, Wade CM, Katz ML, Johnson GC, Taylor JF, Perloski M, Biagi T, Baranowska I, Long S, March PA, Olby NJ, Shelton GD, Khan S, O'Brien DP, Lindblad-Toh K, Coates JR**. Genome-wide association analysis reveals a SOD1 mutation in canine degenerative myelopathy that resembles amyotrophic lateral sclerosis. *Proc Natl Acad Sci USA* 106: 2794-2799, 2009.
4. **Barbera-Cremades M, Baroja-Mazo A, Gomez AI, Machado F, Di Virgilio F, Pelegrin P**. P2X7 receptor-stimulation causes fever via PGE2 and IL-1beta release. *FASEB J* 26: 2951-2962, 2012.
5. **Bartlett R, Yerbury JJ, Sluyter R**. P2X7 receptor activation induces reactive oxygen species formation and cell death in murine EOC13 microglia. *Mediators Inflamm* 2013: 271813, 2013.
6. **Beaucage KL, Xiao A, Pollmann SI, Grol MW, Beach RJ, Holdsworth DW, Sims SM, Darling MR, Dixon SJ**. Loss of P2X7 nucleotide receptor function leads to abnormal fat distribution in mice. *Purinergic Signal* DOI 10.1007/s11302-013-9388-x, 2013.
7. **Bhaskaracharya A, Dao-Ung P, Jalilian I, Spildrejorde M, Skarratt KK, Fuller SJ, Sluyter R, Stokes L**. Probenecid Blocks Human P2X7 Receptor-Induced Dye Uptake via a Pannexin-1 Independent Mechanism. *PLoS One* 9: e93058, 2014.
8. **Boudreaux MK, Martin M**. P2Y12 receptor gene mutation associated with postoperative hemorrhage in a Greater Swiss Mountain dog. *Vet Clin Pathol* 40: 202-206, 2011.
9. **Boyko AR, Quignon P, Li L, Schoenebeck JJ, Degenhardt JD, Lohmueller KE, Zhao K, Brisbin A, Parker HG, vonHoldt BM, Cargill M, Auton A, Reynolds A, Elkahloun AG, Castelhano M, Mosher DS, Sutter NB, Johnson GS, Novembre J, Hubisz MJ, Siepel A, Wayne RK, Bustamante CD, Ostrander EA**. A simple genetic architecture underlies morphological variation in dogs. *PLoS Biol* 8: e1000451, 2010.

10. **Bradley HJ, Browne LE, Yang W, Jiang LH**. Pharmacological properties of the rhesus macaque monkey P2X7 receptor. *Br J Pharmacol* 164: 743-754, 2011.
11. **Browne LE, Compan V, Bragg L, North RA**. P2X7 receptor channels allow direct permeation of nanometer-sized dyes. *J Neurosci* 33: 3557-3566, 2013.
12. **Chessell IP, Simon J, Hibell AD, Michel AD, Barnard EA, Humphrey PP**. Cloning and functional characterisation of the mouse P2X7 receptor. *FEBS Lett* 439: 26-30, 1998.
13. **Constantinescu P, Wang B, Kovacevic K, Jalilian I, Bosman GJ, Wiley JS, Sluyter R**. P2X7 receptor activation induces cell death and microparticle release in murine erythroleukemia cells. *Biochim Biophys Acta* 1798: 1797-1804, 2010.
14. **Denlinger LC, Coursin DB, Schell K, Angelini G, Green DN, Guadarrama AG, Halsey J, Prabhu U, Hogan KJ, Bertics PJ**. Human P2X7 pore function predicts allele linkage disequilibrium. *Clin Chem* 52: 995-1004, 2006.
15. **Donnelly-Roberts DL, Namovic MT, Han P, Jarvis MF**. Mammalian P2X7 receptor pharmacology: comparison of recombinant mouse, rat and human P2X7 receptors. *Br J Pharmacol* 157: 1203-1214, 2009.
16. **Dukes JD, Whitley P, Chalmers AD**. The MDCK variety pack: choosing the right strain. *BMC Cell Biol* 12: 43, 2011.
17. **Fernando SL, Saunders BM, Sluyter R, Skarratt KK, Goldberg H, Marks GB, Wiley JS, Britton WJ**. A polymorphism in the P2X7 gene increases susceptibility to extrapulmonary tuberculosis. *Am J Respir Crit Care Med* 175: 360-366, 2007.
18. **Gadeock S, Pupovac A, Sluyter V, Spildrejorde M, Sluyter R**. P2X7 receptor activation mediates organic cation uptake into human myeloid leukaemic KG-1 cells. *Purinergic Signal* 8: 669-676, 2012.
19. **Gargett CE, Cornish JE, Wiley JS**. ATP, a partial agonist for the P2Z receptor of human lymphocytes. *Br J Pharmacol* 122: 911-917, 1997.
20. **Gargett CE, Wiley JS**. The isoquinoline derivative KN-62 a potent antagonist of the P2Z-receptor of human lymphocytes. *Br J Pharmacol* 120: 1483-1490, 1997.
21. **Gartland A, Skarratt KK, Hocking LJ, Parsons C, Stokes L, Jorgensen NR, Fraser WD, Reid DM, Gallagher JA, Wiley JS**. Polymorphisms in the P2X7 receptor gene are associated with low lumbar spine bone mineral density and accelerated bone loss in post-menopausal women. *Eur J Hum Genet* 20: 559-564, 2012.
22. **Gidlof O, Smith JG, Melander O, Lovkvist H, Hedblad B, Engstrom G, Nilsson P, Carlson J, Berglund G, Olsson S, Jood K, Jern C, Norrving B, Lindgren A, Erlinge D**. A common missense variant in the ATP receptor P2X7 is associated with reduced risk of cardiovascular events. *PLoS One* 7: e37491, 2012.
23. **Gu BJ, Saunders BM, Petrou S, Wiley JS**. P2X7 is a scavenger receptor for apoptotic cells in the absence of its ligand, extracellular ATP. *J Immunol* 187: 2365-2375, 2011.
24. **Gu BJ, Zhang WY, Worthington RA, Sluyter R, Dao-Ung P, Petrou S, Barden JA, Wiley JS**. A Glu-496 to Ala polymorphism leads to loss of function of the human P2X$_7$ receptor. *Journal of Biological Chemistry* 276: 11135-11142, 2001.
25. **Jalilian I, Peranec M, Curtis BL, Seavers A, Spildrejorde M, Sluyter V, Sluyter R**. Activation of the damage-associated molecular pattern receptor P2X7 induces interleukin-1β release from canine monocytes. *Vet Immunol Immunopathol* 149: 86-91, 2012.
26. **Jalilian I, Spildrejorde M, Seavers A, Curtis BL, McArthur JD, Sluyter R**. Functional expression of the damage-associated molecular pattern receptor P2X7 on canine kidney epithelial cells. *Vet Immunol Immunopathol* 150: 228-233, 2012.
27. **Jiang LH, Baldwin JM, Roger S, Baldwin SA**. Insights into the molecular mechanisms underlying mammalian P2X7 receptor functions and contributions in diseases, revealed

by structural modeling and single nucleotide polymorphisms. *Front Pharmacol* 4: 55, 2013.

28. **Jiang LH, Mackenzie AB, North RA, Surprenant A**. Brilliant blue G selectively blocks ATP-gated rat P2X$_7$ receptors. *Mol Pharmacol* 58: 82-88, 2000.

29. **Jindrichova M, Kuzyk P, Li S, Stojilkovic SS, Zemkova H**. Conserved ectodomain cysteines are essential for rat P2X7 receptor trafficking. *Purinergic Signal* 8: 317-325, 2012.

30. **Kathrani A, House A, Catchpole B, Murphy A, German A, Werling D, Allenspach K**. Polymorphisms in the TLR4 and TLR5 gene are significantly associated with inflammatory bowel disease in German shepherd dogs. *PLoS One* 5: e15740, 2010.

31. **Kirkness EF, Bafna V, Halpern AL, Levy S, Remington K, Rusch DB, Delcher AL, Pop M, Wang W, Fraser CM, Venter JC**. The dog genome: survey sequencing and comparative analysis. *Science* 301: 1898-1903, 2003.

32. **Lester S, Stokes L, Skarratt K, Gu B, Silvils K, Lessard C, Wiley JS, Rischmueller M**. Epistasis with HLA DR3 implicates the P2X7 receptor in the pathogenesis of primary Sjogren's syndrome. *Arthritis Res Ther* 15: R71, 2013.

33. **Lindblad-Toh K, Wade CM, Mikkelsen TS, Karlsson EK, Jaffe DB, Kamal M, Clamp M, Chang JL, Kulbokas EJ, Zody MC, Mauceli E, Xie X, Breen M, Wayne RK, Ostrander EA, Ponting CP, Galibert F, Smith DR, deJong PJ, Kirkness E, Alvarez P, Biagi T, Brockman W, Butler J, Chin C-W, Cook A, Cuff J, Daly MJ, DeCaprio D, Gnerre S, Grabherr M, Kellis M, Kleber M, Bardeleben C, Goodstadt L, Heger A, Hitte C, Kim L, Koepfli K-P, Parker HG, Pollinger JP, Searle SMJ, Sutter NB, Thomas R, Webber C, Broad Institute Sequencing Platform, Lander ES**. Genome sequence, comparative analysis and haplotype structure of the domestic dog. *Nature* 438: 803-819, 2005.

34. **Michel AD, Chambers LJ, Walter DS**. Negative and positive allosteric modulators of the P2X7 receptor. *Br J Pharmacol* 153: 737-750, 2008.

35. **Nelson DW, Gregg RJ, Kort ME, Perez-Medrano A, Voight EA, Wang Y, Grayson G, Namovic MT, Donnelly-Roberts DL, Niforatos W, Honore P, Jarvis MF, Faltynek CR, Carroll WA**. Structure-activity relationship studies on a series of novel, substituted 1-benzyl-5-phenyltetrazole P2X7 antagonists. *J Med Chem* 49: 3659-3666, 2006.

36. **North RA, Jarvis MF**. P2X receptors as drug targets. *Mol Pharmacol* 83: 759-769, 2013.

37. **Parker JC, Snow RL**. Influence of external ATP on permeability and metabolism of dog red blood cells. *Am J Physiol* 223: 888-893, 1972.

38. **Pupovac A, Foster CM, Sluyter R**. Human P2X7 receptor activation induces the rapid shedding of CXCL16. *Biochem Biophys Res Commun* 432: 626-631, 2013.

39. **Pupovac A, Stokes L, Sluyter R**. CAY10593 inhibits the human P2X7 receptor independently of phospholipase D1 stimulation. *Purinergic Signal* 9: 609-619, 2013.

40. **Qu Y, Franchi L, Nunez G, Dubyak GR**. Nonclassical IL-1 beta secretion stimulated by P2X7 receptors is dependent on inflammasome activation and correlated with exosome release in murine macrophages. *J Immunol* 179: 1913-1925, 2007.

41. **Rassendren F, Buell G, Newbolt A, North RA, Surprenant A**. The permeabilizing ATP receptor, P2X$_7$. Cloning and expression of human cDNA. *J Biol Chem* 272: 5482-5486, 1997.

42. **Roman S, Cusdin FS, Fonfria E, Goodwin JA, Reeves J, Lappin SC, Chambers L, Walter DS, Clay WC, Michel AD**. Cloning and pharmacological characterization of the dog P2X7 receptor. *Br J Pharmacol* 158: 1513-1526, 2009.

43. **Rosen DR, Siddique T, Patterson D, Figlewicz DA, Sapp P, Hentati A, Donaldson D, Goto J, O'Regan JP, Deng HX, et al.** Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature* 362: 59-62, 1993.

44. **Sluyter R, Shemon AN, Hughes WE, Stevenson RO, Georgiou JG, Eslick GD, Taylor RM, Wiley JS**. Canine erythrocytes express the P2X$_7$ receptor: greatly increased function compared to human erythrocytes. *Am J Physiol Regul Integr Comp Physiol* 293: R2090-R2098, 2007.

45. **Sluyter R, Stokes L**. Significance of P2X7 receptor variants to human health and disease. *Recent Pat DNA Gene Seq* 5: 41-54, 2011.

46. **Spadafora D, Hawkins EC, Murphy KE, Clark LA, Ballard ST**. Naturally occurring mutations in the canine CFTR gene. *Physiol Genomics* 42: 480-485, 2010.

47. **Spildrejorde M, Curtis SJ, Curtis BL, Sluyter R**. Extracellular adenosine 5'-triphosphate and lipopolysaccharide induce interleukin-1β release in canine blood. *Vet Immunol Immunopathol* 157: 105-110, 2014.

48. **Stevenson RO, Taylor RM, Wiley JS, Sluyter R**. The P2X$_7$ receptor mediates the uptake of organic cations in canine erythrocytes and mononuclear leukocytes: comparison to equivalent human cell types. *Purinergic Signal* 5: 385-394, 2009.

49. **Stokes L, Fuller SJ, Sluyter R, Skarratt KK, Gu BJ, Wiley JS**. Two haplotypes of the P2X7 receptor containing the Ala-348 to Thr polymorphism exhibit gain-of-function effect and enhanced interleukin-1β secretion. *FASEB J* 24: 2916-2927, 2010.

50. **Stokes L, Jiang LH, Alcaraz L, Bent J, Bowers K, Fagura M, Furber M, Mortimore M, Lawson M, Theaker J, Laurent C, Braddock M, Surprenant A**. Characterization of a selective and potent antagonist of human P2X7 receptors, AZ11645373. *Br J Pharmacol* 149: 880-887, 2006.

51. **Surprenant A, Rassendren F, Kawashima E, North RA, Buell G**. The cytolytic P2Z receptor for extracellular ATP identified as a P2X receptor (P2X$_7$). *Science* 272: 735-738, 1996.

52. **Syberg S, Petersen S, Beck Jensen JE, Gartland A, Teilmann J, Chessell I, Steinberg TH, Schwarz P, Jorgensen NR**. Genetic background strongly influences the bone phenotype of P2X7 receptor knockout mice. *J Osteoporos* 2012: 391097, 2012.

53. **Vonholdt BM, Pollinger JP, Lohmueller KE, Han E, Parker HG, Quignon P, Degenhardt JD, Boyko AR, Earl DA, Auton A, Reynolds A, Bryc K, Brisbin A, Knowles JC, Mosher DS, Spady TC, Elkahloun A, Geffen E, Pilot M, Jedrzejewski W, Greco C, Randi E, Bannasch D, Wilton A, Shearman J, Musiani M, Cargill M, Jones PG, Qian Z, Huang W, Ding ZL, Zhang YP, Bustamante CD, Ostrander EA, Novembre J, Wayne RK**. Genome-wide SNP and haplotype analyses reveal a rich history underlying dog domestication. *Nature* 464: 898-902, 2010.

54. **Wiley JS, Sluyter R, Gu BJ, Stokes L, Fuller SJ**. The human P2X7 receptor and its role in innate immunity. *Tissue Antigens* 78: 321-332, 2011.

Figure Legends

Fig. 1. Relative monocyte P2X7 function varies between dogs. PBMCs (from 52 dogs as indicated) or MDCK cells in NaCl medium containing 1 μM YO-PRO-1^{2+} were incubated in the absence or presence of 1 mM ATP for 5 min at 37°C. Incubations were stopped by addition of MgCl$_2$ medium and centrifugation, and YO-PRO-1^{2+} uptake into CD14$^+$ monocytes or MDCK

cells determined by flow cytometry. ATP-induced YO-PRO-1^{2+} uptake was defined as the mean difference (from duplicate samples) in mean fluorescent intensity in the presence and absence of ATP for each individual. Symbols represent individual dogs; bars represent group means; broken line represents mean of all samples.

Fig. 2. The F103L, R270C and R365Q SNPs correspond to decreased monocyte P2X7 function. *A*: Sequence analysis of the canine *P2RX7* gene identified four missense SNPs as indicated. The L440F SNP identified in the cloned English Springer Spaniel P2X7 (but absent in all other dogs sequenced) is also shown. Boxes represent *P2RX7* exons (not all 13 exons are represented). *B*: For each dog, the *P2RX7* genotype (as indicated) was compared to the relative monocyte P2X7 function (obtained in Fig. 1). *$P<0.05$ to corresponding wild-type. Symbols represent individual dogs; bars represent group means.

Fig. 3. Characterization of P2X7 cloned from an English Springer Spaniel. *A*: Sequence analysis of the P2X7 cloned from an English Springer Spaniel revealed two missense SNPs, 440F and 452S, but was wild-type for F103, R270 and R365 as shown. Boxes represent *P2RX7* exons (not all 13 exons are represented). *B*: Whole lysates of EOC13 cells, HEK-293 cells transfected with English Springer Spaniel P2X7 plasmid DNA or mock-transfected HEK-293 cells were examined by immunoblotting using an Ab against either the (*left panel*) extracellular epitope or (*right panel*) C-terminal epitope of P2X7. *C-F*: HEK-293 cells transfected with English Springer Spaniel P2X7 plasmid DNA or mock-transfected HEK-293 cells were suspended in NaCl medium containing (*C-F*) 25 μM ethidium^{+}, (*D*) 1 μM YO-PRO-1^{2+} or 25 μM propidium^{2+} at 37°C. (*C, D*) Cells were incubated in the absence (basal) or presence of 1 mM ATP for 5 min. *E*: Cells were incubated in the absence or presence of ATP, BzATP or ATPγS as indicated, or 1 mM ADP, UTP or αβ-meATP for 5 min. *F*: Cells were preincubated in the absence or presence

of A438079, AZ10606120, AZ11645373, BBG or KN-62 as indicated for 15 min, before addition of ethidium$^+$, and incubation in the absence or presence of 250 μM ATP (equivalent to the EC$_{50}$ for ATP) for 5 min. *C-F*: Incubations were stopped by addition of MgCl$_2$ medium and centrifugation, and cation dye uptake determined by flow cytometry. Cation dye uptake is expressed as (*C, D*) mean fluorescent intensity, (*E*) percent of maximum response to 50 μM BzATP or (*F*) percent of maximum response to 250 μM ATP in the absence of antagonist. Results are mean (SD), *n*=3; **P<0.01 to corresponding basal.

Fig. 4. The R270C SNP results in loss of function of canine P2X7. *A-C*: HEK-293 cells, transfected with wild-type or mutant canine P2X7 plasmid DNA (as indicated), suspended in NaCl medium containing 25 μM ethidium$^+$ were incubated in the absence (basal) or presence of 1 mM ATP for 5 min at 37°C. Incubations were stopped by addition of MgCl$_2$ medium and centrifugation. Ethidium$^+$ uptake was determined by flow cytometry and expressed as mean fluorescent intensity. Results are mean (SD), *n*=3-4; **P<0.01 to corresponding basal, **P<0.01 to (*A*) 440F/452S or (*B, C*) corresponding wild-type. *D-F*: Whole lysates of HEK-293 cells transfected with wild-type or mutant canine P2X7 plasmid DNA (as indicated) were examined by immunoblotting using an Ab against the extracellular epitope of P2X7.

Fig. 5. The R270C SNP results in loss of canine P2X7 channel activity. HEK-293 cells were transfected with wild-type (*left panel*), 452S mutant (*middle panel*) or 270C/452S (*right panel*) canine P2X7 plasmid DNA. Cells were clamped at -60 mV at room temperature, 1 mM ATP in low divalent solution was applied using a fast-flow delivery system and whole cell currents were recorded. (A) Inward current to first exposure to ATP and (B) repeated exposures to ATP every 60 sec. Black bars indicate ATP exposure (5 sec). Traces are representative of five to seven cells.

Fig. 6. Canine P2X7 containing the R270C SNP is expressed on the cell-surface of transfected HEK-293 cells. HEK-293 cells transfected with wild-type (*top panel*), 452S mutant (*middle panel*) or 270C/452S mutant (*bottom panel*) canine P2X7 plasmid DNA were examined by immunolabeling with an Ab specific for the extracellular loop of P2X7 and confocal microscopy.

Fig. 7. SNPs of canine P2X7. P2X7 protein alignment of sequences from five species illustrating the relative position of (bold) five missense SNPs and one synonymous SNP identified in canine P2X7 (canine P2X7 numbering). (Highlighted grey) Missense SNPs identified in human (R270H) and mouse (P451L, mouse P2X7 numbering) P2X7 in equivalent positions to 270 and 452 in canine P2X7 are also shown. The sequences correspond to the following accession numbers or references: NM_001113456 (canine, NCBI, National Center for Biotechnology Information); XM_534669 (canine, Boxer); Ref. (42) (canine, GSK, GlaxoSmithKline); and the current study (canine, EES, English Springer Spaniel); Y09561 (human); Ref. (10) (rhesus macaque); X95882 (rat); AJ009823 (mouse).

Table 1. *Primers used to amplify and sequence the canine P2RX7 gene*

Exon	Forward Primer (5'-3')	Reverse primer (5'-3')	Size (bp)*	Temp ($^{\circ}$C)[†]
1	AGGATTGAACCCTCCTCGTT	TCAAGATGTGCACCAGAAGC	263	58.7
2	AAGTAGCCTGCCCAGAGTCA	TTTTGAGCTGCTGGATCTGA	486	64.1
3	AGTCCAAGTTGCTCCCAGAC	GAGGACAGAAGAGGGAAAAGA	349	63.1
4	TCCTGTTGTAGCAGGTGCAG	GCTGAGAGCCAGAGGCTAAA	423	64.1
5	AAAAAGCCAACAGCATCCAC	CTCTTCCTCCCCTGCTCTTT	741	60.0
6	GGGCCTCAGGAAGAGAGAAG	CCCACAGAAACTGCATCAGA	346	64.5
7	CATGGAACCCAGCTACCAAC	GCACTGTGCCGGGATATACT	353	64.1
8	TTTAATCTGCGTTCCCCTCT	AAGCCTTTTGCATTCATCTTG	368	63.1
9	TGAAATACCAGCATGTTCAC	GGACATTTCTTCCCCCTCTT	263	58.7
10	GCTGACAATTACATGGTAGAGCA	CTAAAGTGTAGGAAAGAGAA	170	62.2
11	CTCACTAACTCGCACTGAT	TGATTTTGGCCTTATTTTTGC	262	62.2
12	GAATAACACTTGCCTTTAGATATGTTC	TGGGTTACATGTACTTAATGTGTTAAA	300	60.0
13[‡]	GAACCTTAGGGGTGGTCACT	CAAGCTCAGGTGCAAACAAA	600	64.1
13[‡]	TTTCAGAGACCCTCAATGGAA	TCAACCCTGTGCCATCAGTA	515	58.6

*Size of amplicon (bp, base pairs)
[†]Annealing temperatures used for amplification of exons
[‡]Two overlapping primer pairs were used to amplify exon 13.

Table 2. *P2RX7 genotype and P2X7 function of dogs*

Breed	P2RX7 Genotype*				P2X7 Function[†]
	F103L	R270C	R365Q	P452S	
Alaskan Malamute	WT	WT	WT	P/S	192
Alaskan Malamute	WT	WT	WT	P/S	263
Alaskan Malamute and Siberian Husky cross		WT	WT	P/S	187
American Staffordshire Terrier	F/L	WT	WT	WT	195
American Staffordshire Terrier	F/L	WT	WT	P/S	85
American Staffordshire Terrier	L/L	WT	WT	WT	77
Australian Bulldog	L/L	WT	WT	WT	207
Australian Cattle Dog	F/L	WT	WT	P/S	282
Australian Kelpie		WT	WT	P/S	173
Basenji	WT	WT	WT	WT	258
Beagle	WT	WT	WT	WT	
Beagle	WT	WT	WT	S/S	165
Border Collie	WT	WT	WT	P/S	149
Boarder Collie and Australian Cattle Dog cross	WT	WT	WT	WT	395
Bull Terrier	L/L	WT	WT	WT	
Bull Terrier cross	F/L	WT	WT	WT	256
Bull Terrier (Miniature)	L/L	WT	WT	WT	165
Cavalier King Charles Spaniel	WT	WT	WT	S/S	129
Cavalier King Charles Spaniel and Cocker Spaniel cross	WT	R/C	WT	S/S	44
Chihuahua and Dachshund (Miniature) cross	F/L	WT	WT	WT	130
Corgi cross	F/L	WT	WT	P/S	
Dachshund	WT	WT	WT		
Dachshund	WT			WT	
Dachshund (Miniature)	WT	WT	WT	S/S	82
Dachshund (Miniature)	WT	WT	WT	S/S	129
Dachshund (Miniature)		WT	WT	S/S	168
Dachshund (Miniature)	L/L	WT	WT	S/S	30
Fox Terrier (Miniature)	WT	WT	WT	P/S	
Fox Terrier (Miniature)	F/L	WT	WT	P/S	123
German Shorthaired Pointer	WT	WT	WT	S/S	
Golden Retriever	WT	WT	WT	P/S	153
Golden Retriever and Labrador Retriever cross	L/L	WT	R/Q	WT	31
Jack Russell Terrier	WT	WT	WT	P/S	106
Jack Russell Terrier	L/L	WT	WT	WT	32
Jack Russell Terrier cross	L/L	WT	WT	WT	
Jack Russell Terrier cross	L/L	WT	WT	WT	
Labrador Retriever	WT	WT	WT	WT	152
Labrador Retriever	WT	WT	WT	P/S	21
Labrador Retriever	WT	WT	WT	P/S	272
Labrador Retriever	WT	WT	R/Q	P/S	
Labrador Retriever	WT	WT	R/Q	P/S	80
Labrador Retriever		WT	R/Q	S/S	196
Maltese	WT	WT	WT	WT	58
Maltese cross	F/L	WT	WT		
Maltese and Poodle cross	F/L			WT	
Maltese and Shih Tzu cross	WT	WT	WT	P/S	126
Maltese and Shih Tzu cross	WT	WT	WT	P/S	208
Maltese and Shih Tzu cross	WT	WT	WT	S/S	246
Mastiff and American Staffordshire Bull Terrier cross	L/L	WT	WT	WT	64
Poodle and Cocker Spaniel cross	WT	WT	WT	S/S	
Pug and Beagle cross	L/L	WT	WT	S/S	35
Pug and Fox Terrier cross					49
Pug and Pomeranian cross	F/L	WT	WT	P/S	80
Rhodesian Ridgeback	WT	WT	WT	P/S	236
Rhodesian Ridgeback and Australian Cattle Dog cross	WT	WT	WT		
Schnauzer (Miniature) and Bulldog cross	F/L	WT	WT	P/S	88
Schnauzer (Miniature) and Bulldog cross	L/L	WT	WT	WT	122
Shar Pei	WT	WT	WT	P/S	337
Siberian Husky	WT	WT	WT	P/S	25
Siberian Husky	WT	WT	WT	P/S	466
Staffordshire Bull Terrier	L/L	WT	WT	WT	
Staffordshire Bull Terrier	L/L	WT	WT	WT	
Staffordshire Bull Terrier	L/L	WT	WT	WT	64
Staffordshire Bull Terrier	L/L	WT	WT	WT	165
Staffordshire Bull Terrier	L/L	WT	WT	WT	146
Staffordshire Bull Terrier cross		WT	WT	WT	198
Staffordshire Bull Terrier cross	F/L	WT	WT	P/S	
Staffordshire Bull Terrier cross	F/L	WT	WT	P/S	223
Staffordshire Bull Terrier cross	L/L	WT	WT	P/S	39
MDCK cells (Cocker Spaniel)	WT	R/C	WT	P/S	0

*P2RX7 genotype was determined by sequencing of genomic DNA.
[†]P2X7 function was determined by flow cytometric measurements of ATP-induced YO-PRO-1^{2+} uptake into CD14$^+$ monocytes (or MDCK cells).
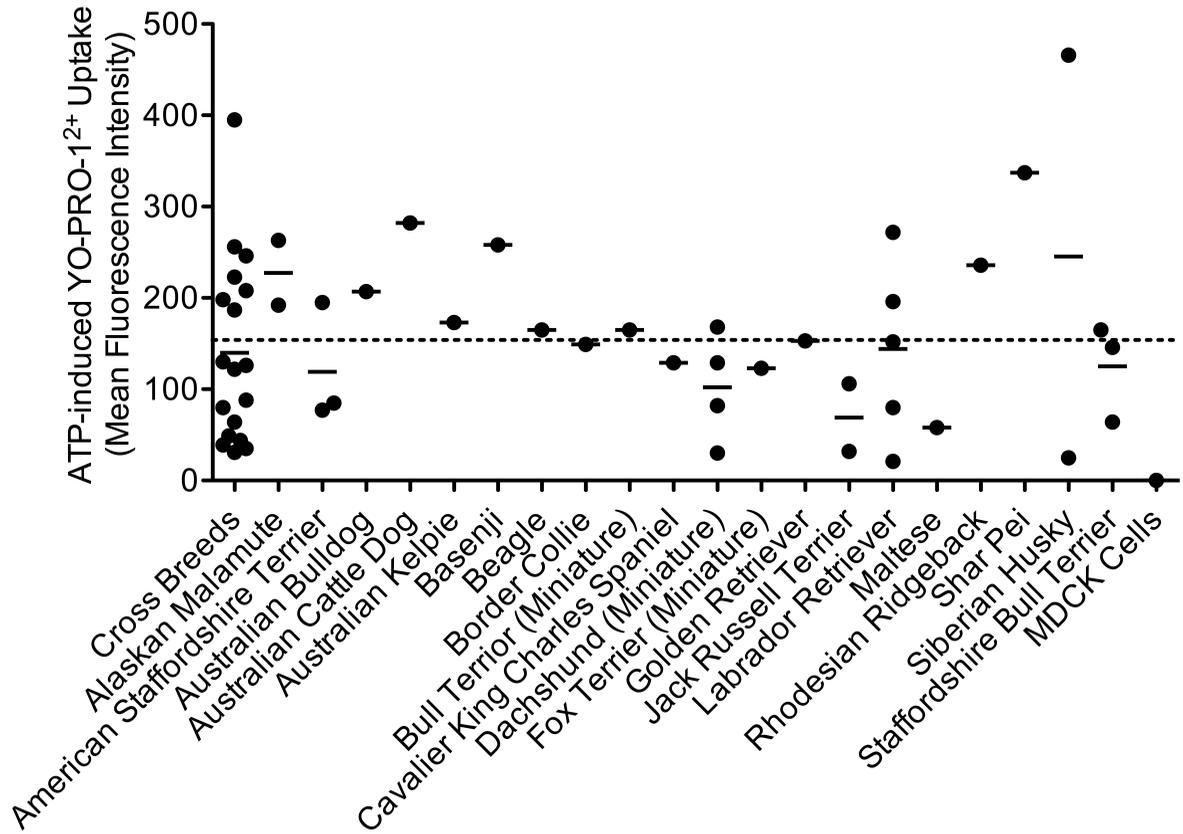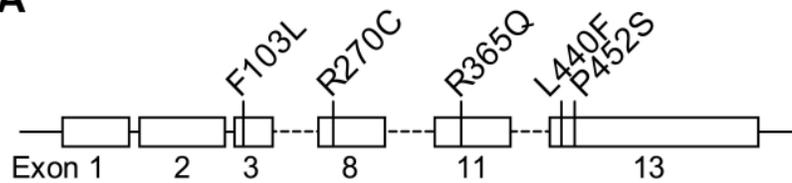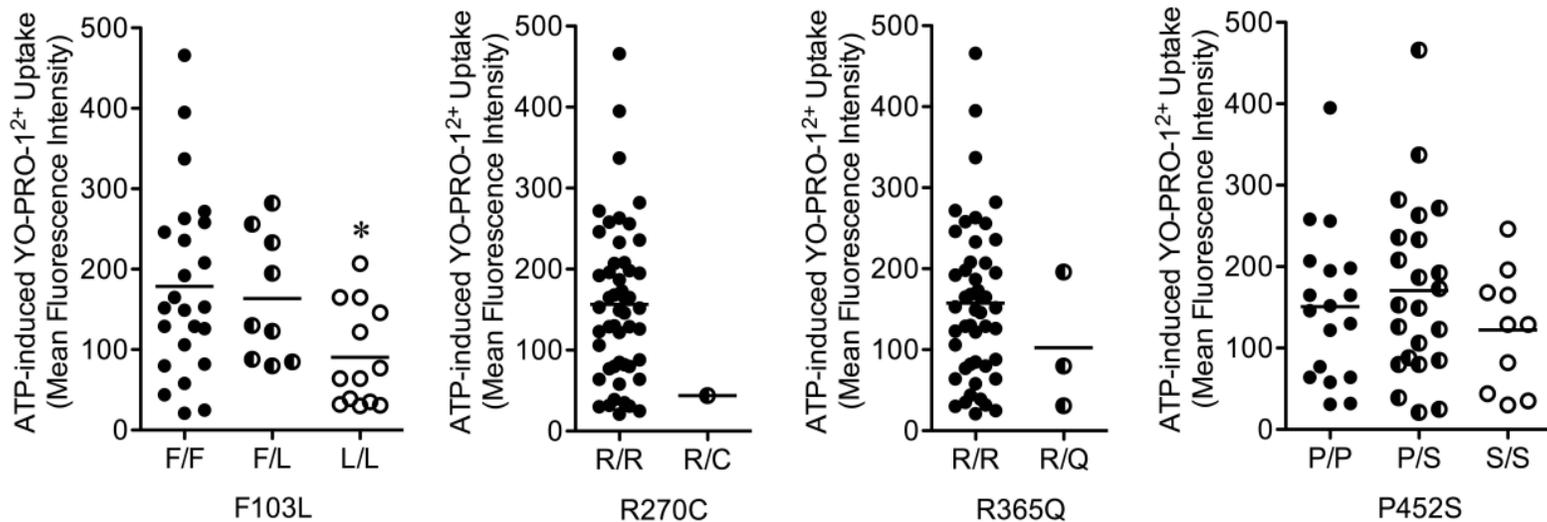
Figure 1

Figure 2

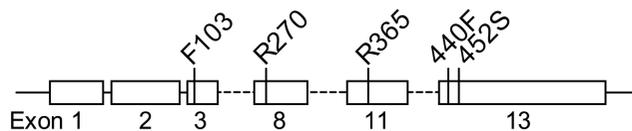# Figure 3

Figure 4

Wild-type   **452S**   **270C/452S**

A

200pA

5 secs

B

200pA

5 secs
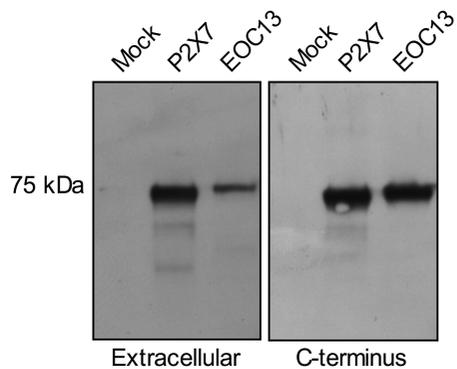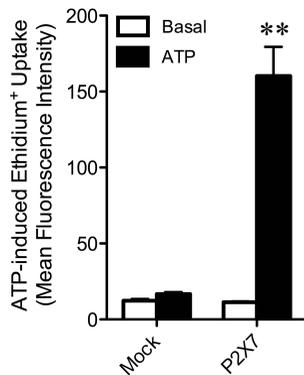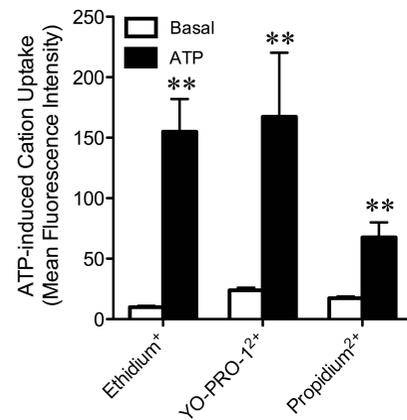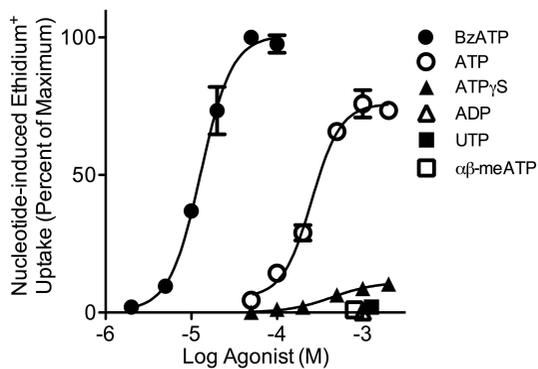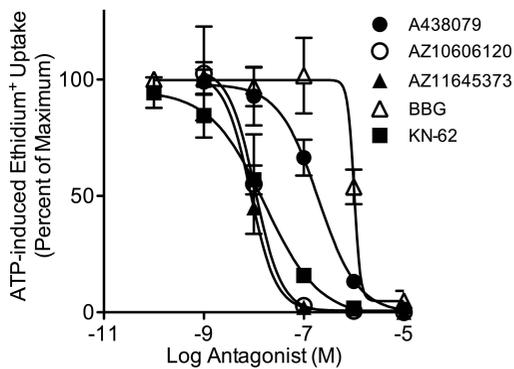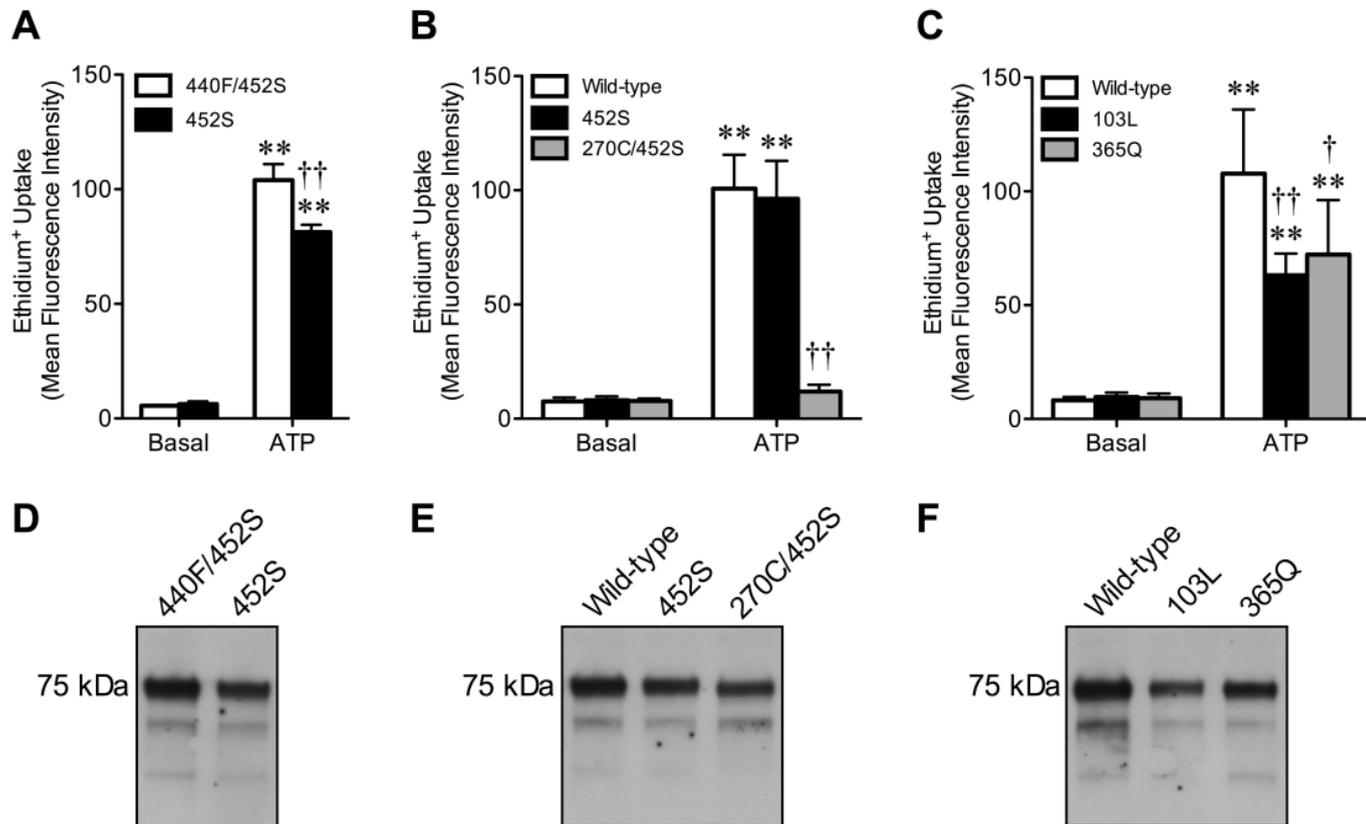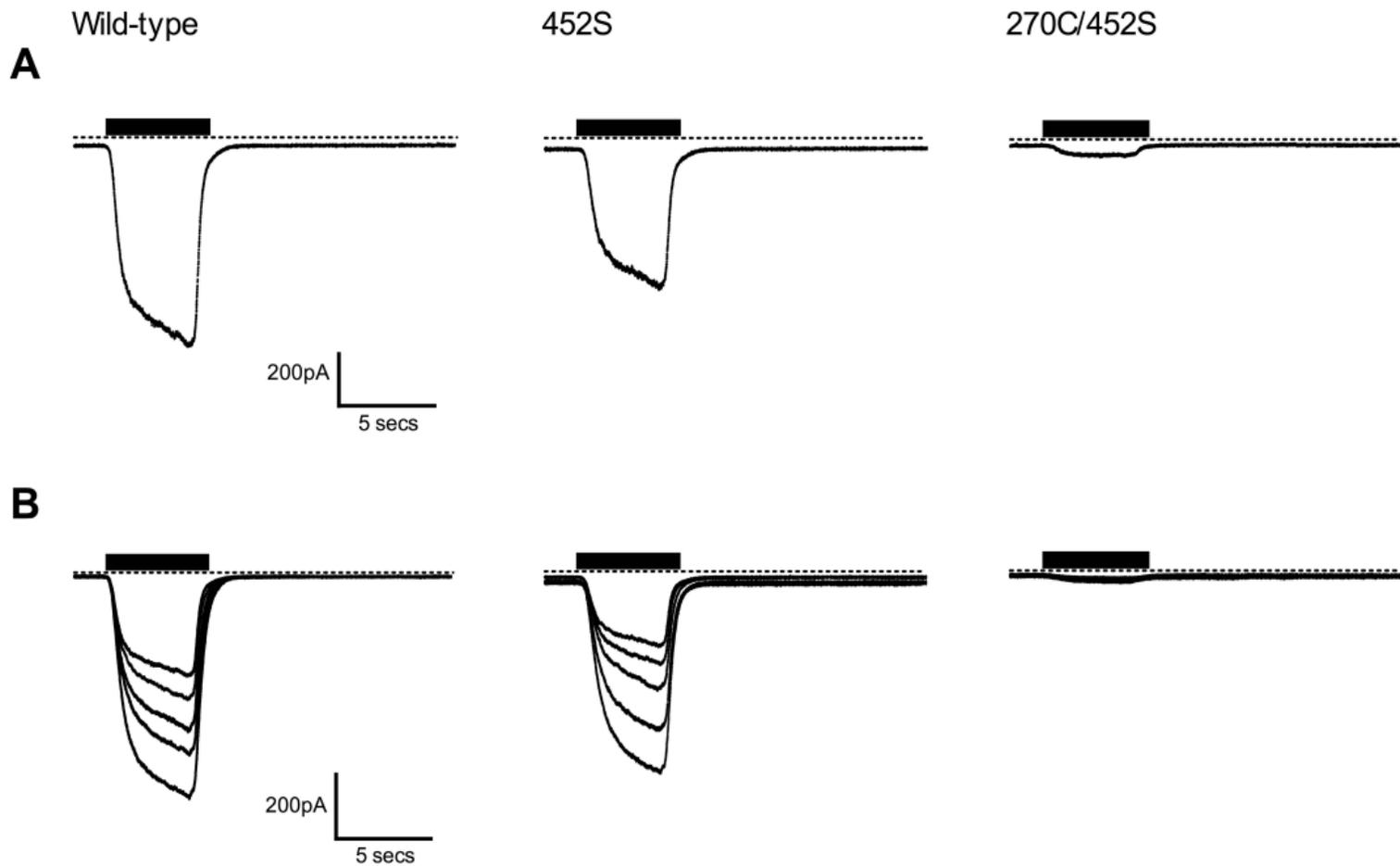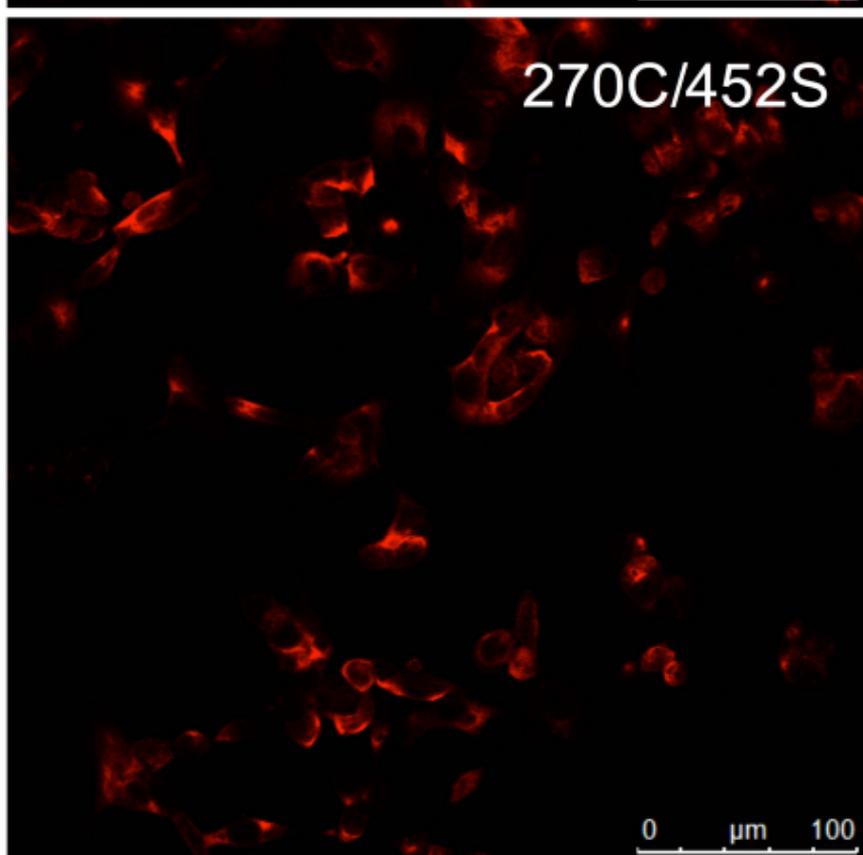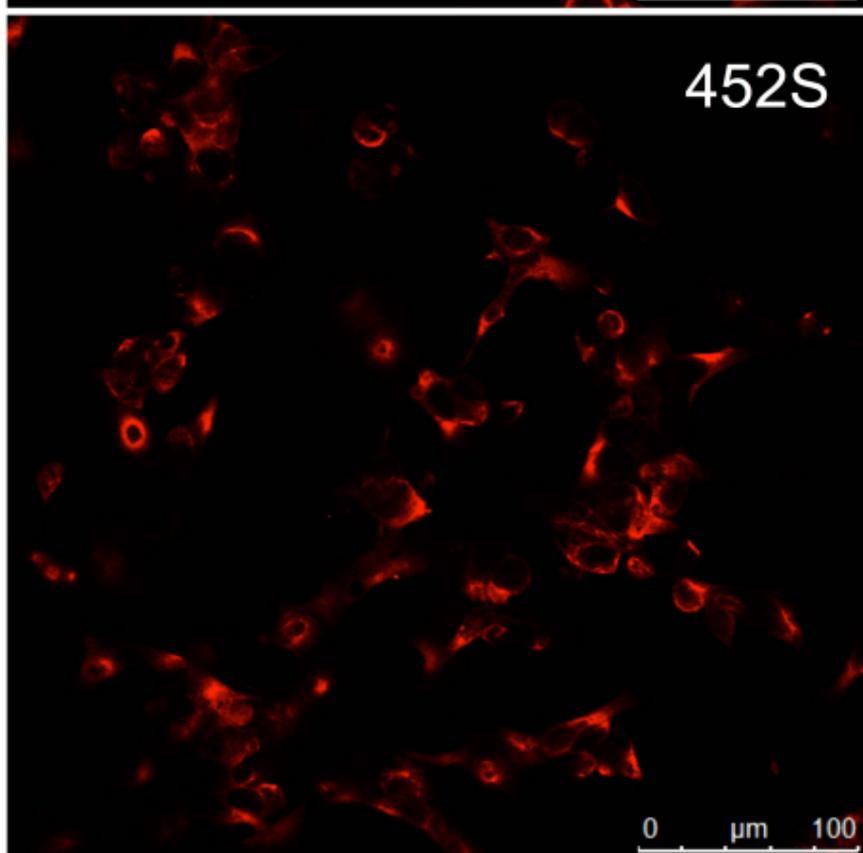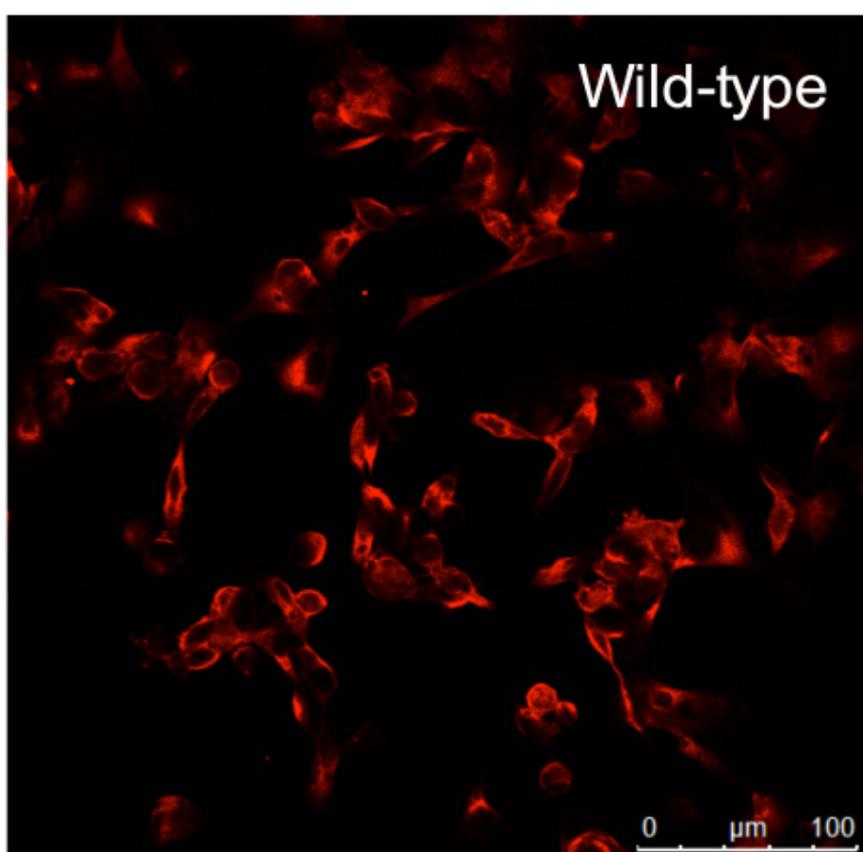
Figure 6

Figure 7

| | **F103L** | **N131N** | **R270C** | **R365Q** | **L440F** | **P452S** |
| | \| | \| | \| | \| | \| | \| |

Canine, NCBI   ···NSF**F**VMT···LCS**N**DWG···HHC**R**PKY···KCC**R**SHI···LSR**L**SLS···DLS**P**IPG···
Canine, Boxer   ···NSF**L**VMT···LCS**N**DWG···HHC**R**PKY···KCC**R**SHI···LSR**L**SLS···DLS**P**IPG···
Canine, GSK   ···NSF**L**VMT···LCS**N**DWG···HHC**R**PKY···KCC**R**SHI···LSR**L**SLS···DLS**P**IPG···
Canine, EES   ···NSF**F**VMT···LCS**N**DWG···HHC**R**PKY···KCC**R**SHI···LSR**F**SLS···DLS**S**IPG···
Human   ···NSF**F**VMT···LCS**S**DRG···HHC**R**PKY···NCC**R**SHI···LSR**L**PLA···DTP**P**IPG···
Rhesus Macaque ···NSF**F**VMT···LCS**S**DRG···HHC**R**PKY···NYC**R**SHI···LSK**L**PLA···DPP**P**IPG···
Rat   ···NSF**F**VMT···QCH**S**DQG···HRC**Q**PKY···TCC**R**SRV···LSR**L**SLS···HSP**P**IPG···
Mouse   ···NSF**F**VMT···QCS**S**DRR···HHC**R**PKY···AFC**R**SGV···LSR**L**SLS···DSP**P**TPG···