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Supplemental Information (SI)

Title: P2Y$_{12}$-R Localizes in Rat Renal Collecting Duct, and its Blockade Augments AVP Action and Alleviates Lithium-induced NDI

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Materials and Methods:

Rationale for the Dose of Clopidogrel Used: Drug dosage translation across species is not straightforward, and is governed by certain factors, such as body surface area (BSA) and metabolic activity of the species among others[1]. Metabolic activity of the species is known to influence the pharmacokinetics of drugs. Due to these inherent qualities of the species, often a dose that works well in humans is ostensibly ineffective in animals, and a dose that works well in animals may be very toxic in humans (e.g., the toxicity of aminoglycosides and cisplatin on the kidney). We applied the following formula for dose translation based on BSA, which is used while developing new drugs for clinical use[2].

Formula for Dose Translation Based on BSA

$$\text{HED (mg/kg)} = \text{Animal Dose (mg/kg)} \times \frac{\text{Animal Km}}{\text{Human Km}}$$

HED of clopidogrel is 75 mg/day = 1.25 mg/kg bw/day.

Species	Weight (kg)	Body Surface Area (BSA) (m^2)	K_m factor Weight (kg) / BSA (m^2)
Adult Human	60	1.6	37
Rat	0.15	0.025	6
Mouse	0.02	0.007	3

Values based on data from FDA Draft Guidelines (2002), cited by Reagan-Shaw et al[2]

By substituting the 20 mg/kg dose we gave to Sprague-Dawley rats as well as the corresponding K_m values on the right side of the above equation we get a value of 3.24 on the left side, which is 2.6-fold higher than the HED (human effective dose) of clopidogrel. A higher dose of clopidogrel (40 mg/kg bw/day) was used in Brattleboro rats to ensure an unequivocal outcome, whether positive or negative. The dose of clopidogrel given in the wild type and P2Y$_2$-R knockout mice (80 mg/kg

bw/day) is approximately 5-fold higher than the HED. This higher dose ensured detection of off-target effects of clopidogrel, if any, on $P2Y_2$ receptor. Furthermore, considering the fact that generally rodents, which have a higher metabolic activity, are resistant to many drugs the doses we gave are within the range used for animal studies. Furthermore, toxicological evaluation of clopidogrel showed that doses as high as 165 mg/kg bw/day for up to 4 weeks in rats were free from toxicity[3].

Composition of Culture Media Used for Primary Cultures of Rat IMCD Cells: The modified defined medium consists of DMEM/Ham's F12 (1:1 vol/vol), 7 ng/ml sodium selenite, 5 µg/ml transferrin, 5 µg/ml insulin, 100 units/ml penicillin, 100 µg/ml streptomycin, 0.29 mg/ml L-glutamine, 5 nM triiodthyronine, 50 nM hydrocortisone, 15 ng/ml epidermal growth factor (EGF), 10 U/ml recombinant rat interferon-γ, and 10 µM dibutyryl cyclic AMP. The starvation medium consists of DMEM/Ham's F12 (1:1 vol/vol) and all the above, except EGF and interferon-γ.

Lysates Human Platelets: Dr. Andrew Weyrich laboratory (University of Utah, Salt Lake City, UT) kindly prepared and supplied whole cell lysates of human platelets. These were obtained in accordance with the Declaration of Helsinki with the approval of University of Utah Institutional Review Board (IRB). As previously described[4,5], washed platelets were freshly isolated from healthy subject who consented to participate in the studies. Contaminating leukocytes were removed from the platelet preparations by CD45-positive selection, and purified platelets were lysed in RIPA buffer containing protease and phosphatase inhibitors.

Primary Cultures of Mouse Microglial Cells: Moue microglial cultures were prepared from as described previously[6]. Briefly, brains were removed from 1 day old CD1 mice and cerebral cortical cultures were prepared after trypsinization of the tissue. Cells were seeded in plates pre-coated with poly-D-lysine in MEM containing 10% fetal bovine serum. Culture were grown for 7-9 days at

37°C, and the microglial cells were removed by shaking on an orbital shaker followed by centrifugation. Microglial cells thus obtained were suspended in neurobasal medium with 2% B27 supplement and plated on poly-D-lysine coated plates. For immunofluorescence microscopy, cells were rinsed and fixed in 2% paraformaldehyde in PBS, and permeabilized with 0.2% triton-X 100. Cells were treated with Image-it FX signal enhancer and prior to incubation with primary antibodies. Rat monoclonal antibody for CD11b was used to visualize microglia. Cells were incubated with combination of primary antibodies at 4°C overnight followed by fluorochrome-conjugated secondary antibodies. Propidium iodide was used to visualize cell nuclei. Sections were mounted with ProLong Gold anti-fade permanent mounting media. Personal Confocal Microscope PCM-2000 (NIKON) was use to acquire the images.

Primary Cultures of Rat Hypothalamic Cells: Clumps of live primary rat hypothalamic cells from micro-surgically dissected brains of day 18 embryonic rats were obtained from Neuromics (Edina, MN). Neuromics prepares these cells freshly and ships them in a nutrient rich medium that keeps the cells alive for up to 7 days under refrigeration. Immediately after receiving, the cells were processed as per the instructions provided by the Neuromics. Briefly, the cells were enzymatically disrupted by incubating with papain and mechanically dispersed by repeatedly passing through the tip of a sterile Pasteur pipette. Cells were collected by centrifugation, suspended in Neurobasal medium containing B27 and glutamine, and seeded into poly-D-lysine coated multi-well plates. Cells were incubated at 37°C for up to 7 days while changing the medium every 3rd day. On day 7, test agents, namely 2MeS-ADP (2-methylthio-adenosine diphosphate) and/or PSB-0739 were added to the culture wells and incubated for another 24 h. Thereafter, medium was removed and cells were collected into Trizol reagent for total RNA extraction.

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Attachments:

Table S1: Alignment of amino acid sequences of $P2Y_{12}$ receptor and related proteins with respect to the location of the immunizing peptide.

Table S2: Nucleotide sequences of primer pairs used in real-time PCR

Fig S1: Comparative evaluation of different $P2Y_{12}$-R antibodies on human platelet preparations

Fig S2: Comparative immunofluorescence evaluation of specificity of $P2Y_{12}$-R antibodies in microglial cells.

Fig S3: Immunofluorescence labeling of $P2Y_{12}$-R in rat kidney using Alomone Labs' antibody.

Fig S4: Expression of $P2Y_{12}$-R in hypothalamus and the effect of its blockade on AVP gene expression.

Fig S5: Effect of clopidogrel administration for 13 days in Sprague-Dawley rats.

Fig S6: Lack of interaction of clopidogrel with $P2Y_2$ receptor.

Fig S7: Lack of effect of clopidogrel in Brattleboro rats.

Table S1: Alignment of amino acid sequences of $P2Y_{12}$ receptor and related proteins with respect to the location of the immunizing peptide

Protein	AA Residue Position	Amino Acid (AA) Residues																AA Residue Position
$P2Y_{12}$ mouse	332	K	K	K	G	Q	E	G	G	E	P	S	E	E	T	P	M	347
$P2Y_{12}$ rat	328	K	K	K	G	Q	E	G	G	D	P	S	E	E	T	P	M	343
$P2Y_{13}$	338	Q	–	–	–	E	N	H	S	S	Q	T	D	N	I	T	L	353
$P2Y_{14}$	321	S	K	T	K	R	E	–	–	N	A	I	H	E	S	T	D	336
GPR87 mouse	343	Q	S	V	R	R	S	E	V	R	I	Y	Y	D	Y	T	D	358
GPR171 mouse	306	S	K	P	L	E	E	E	R	L	R	S	E	N	D	V		319
GPR34 mouse	348	S	T	S	E	F	K	P	G	H	S	L	H	L	S	V	K	365

Shaded cells indicate identical amino acid residues with respect to mouse $P2Y_{12}$ receptor.

Table S2: Nucleotide sequences of primer pairs used in real-time PCR

Gene	Accession No.	Primer Position	Primer Sequence	Amplicon Size, bp	Ref.
$P2Y_1$	NM_012800	1235-1254 1504-1523	ACGTCAGATGAGTACCTGCG CCCTGTCGTTGAAATCACAC	289	7, 8, 9
$P2Y_2$	NM_017255	1270-1293 1376-1399	ACCCGCACCCTCTATTACTCCTTC AGTAGAGCACAGGGTCAAGGCAAC	130	10, 11
$P2Y_4$	NM_031680	263-284 537-556	TGTTCCACCTGGCATTGTCAG AAAGATTGGGCACGAGGCAG	294	7, 8, 9
$P2Y_6$	NM_057124	644-665 960-982	TGCTTGGGTGGTATGTGGAGTC TGGAAAGGCAGGAAGCTGATAAC	339	7, 8, 9
$P2Y_{12}$	NM_022800.1	494-517 548-569	TAACCATTGACCGATACCTGAAGA ATCTTCGCACCCAAAAGATTGC	76	Modified from 12
AQP2	BC128705.1	414-434 609-590	ACCT GGCT GTCA ATGC TCTC CCGG TGAA ATAG ATCC CAAG	176	#
AQP3	BC127490.1	96-115 226-245	AGATGCTCCACATCCGCTAC AAGCCAAGTTGATGGTGAGG	150	#
AVP	NM_016992.2	1-21 129-149	CAACACTACGCTCTCTGCTTG TCTCAGCTCCATGTCGGATGT	150	13
β-Actin	NM_031144.2	18-37 205-224	CACCCGCGAGTACAACCTTC CCCATACCCACCATCACACC	207	9

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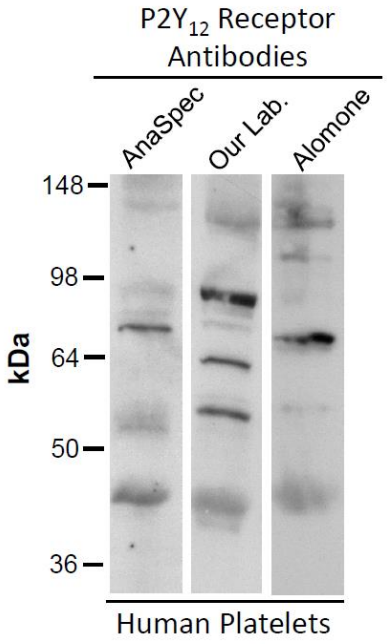

Fig. S1. Comparative evaluation of our $P2Y_{12}$-R antibody with two commercial antibodies (AnaSpec, Inc. and Alomone Labs) on solubilized homogenates of human platelets in immunoblotting.

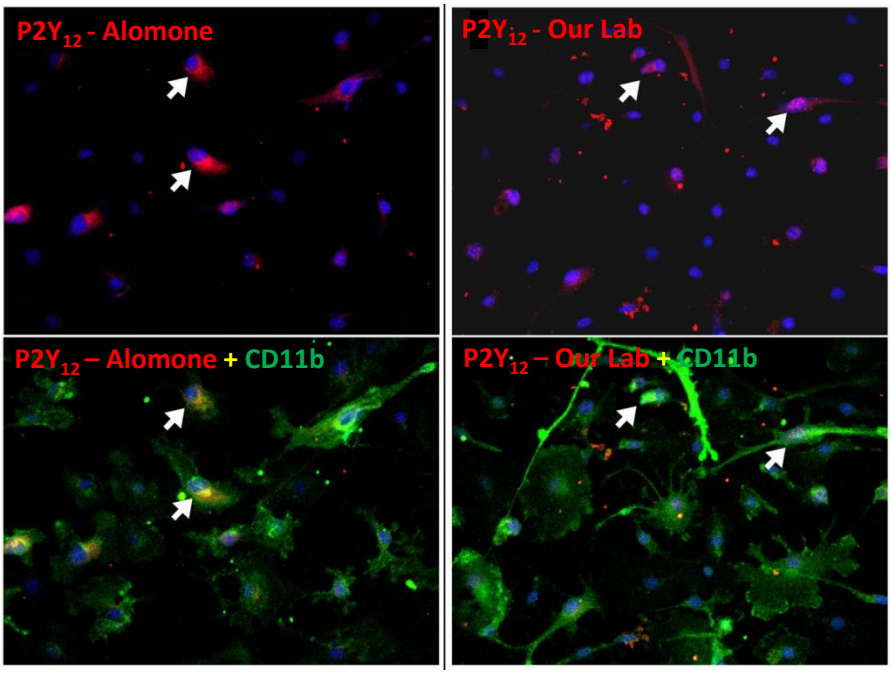

Fig. S2. Comparative immunofluorescence evaluation of specificity of $P2Y_{12}$-R antibodies in microglial cells. Primary cultures of mouse microglial cells were incubated with antibodies against $P2Y_{12}$-R and microglial marker CD11b. Upper row panels show labeling for $P2Y_{12}$-R only (red). Lower row panels show merged images for $P2Y_{12}$-R and CD11b (green). Antibody from the Alomone Labs was used in the left column panels. Right column panels were incubated with our $P2Y_{12}$-R antibody. Nuclei are stained with propidium iodide. Arrows indicate $P2Y_{12}$-R labeled cells in the upper and lower panels for each antibody.

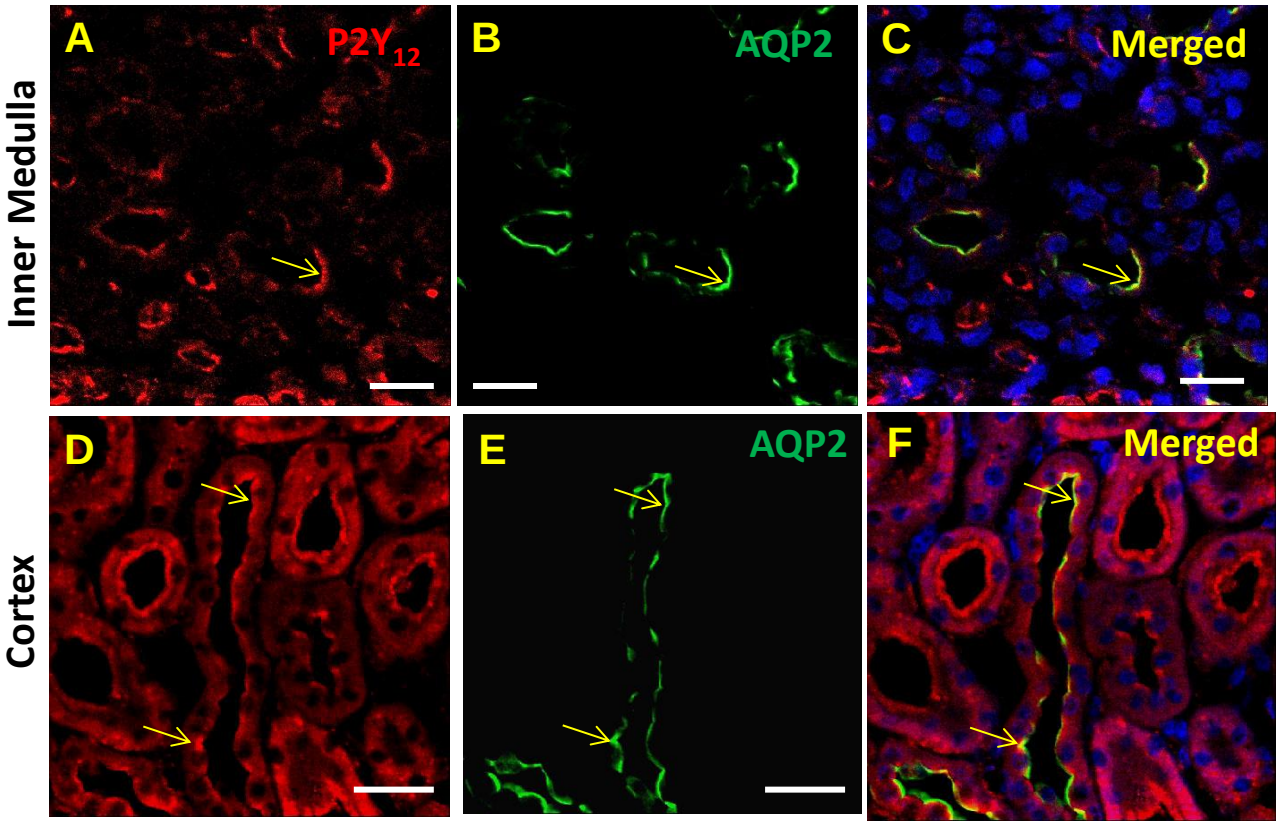

Fig. S3. Immunofluorescence labeling of $P2Y_{12}$-R in rat kidney using Alomone Labs' antibody. (A-C) show labeling for $P2Y_{12}$-R (red), AQP2 (green) or merging of both proteins (yellow) in medullary collecting ducts. (D) show labeling for $P2Y_{12}$-R in proximal tubules, and a cortical collecting duct (arrows). (E) shows labeling for AQP2 in the cortical collecting duct of the same profile as in (D). (F) shows overlay of panels D & E showing merger of labeling for $P2Y_{12}$-R and AQP2 in the cortical collecting duct (arrows). (Nuclei were stained with DAPI. Bars are 20 μm in length.

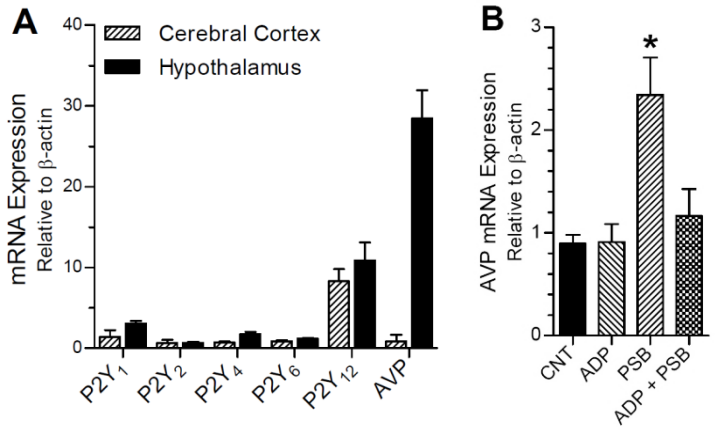

Fig. S4. Expression of $P2Y_{12}$-R in rat hypothalamus and the effect of its blockade on AVP gene expression in hypothalamic cells. (A) Real-time RT-PCR results showing the mRNA expression of key P2Y receptor subtypes and AVP relative to the expression of β-actin in the cerebral cortex and hypothalamus (n = 3 rats). (B) mRNA expression of AVP in the primary cultures of rat hypothalamic cells exposed to PSB-0739 (100 nM) or a combination of PSB-0739 and 2MeS-ADP (50 μM) for 24 hours. N = 5 culture wells per condition. *significantly different ($P < 0.01$ or 0.05) from all other groups. 2MeS-ADP – 2-methylthio ADP, is an agonist of $P2Y_{12}$-R.

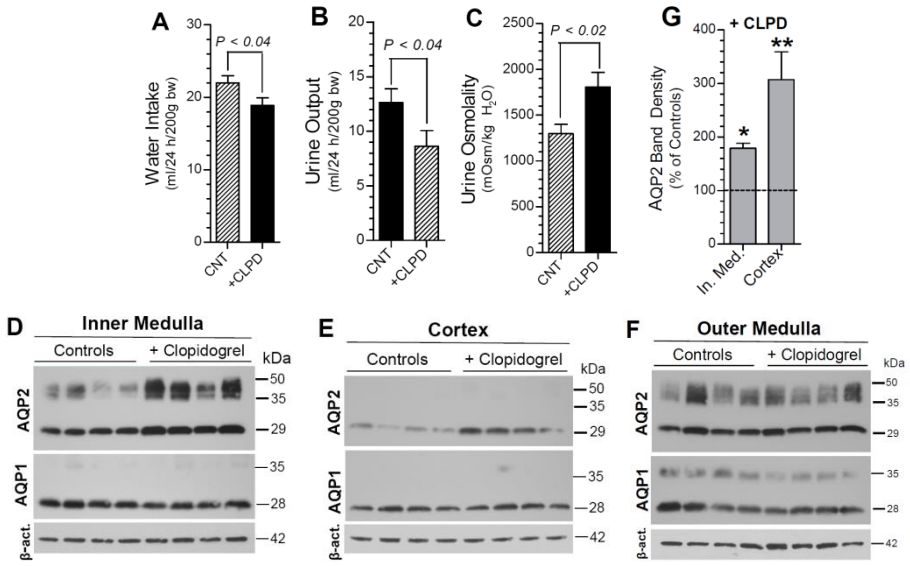

Fig. S5. Effect of clopidogrel (CLPD) administration for 13 days in Sprague-Daley rats. (A) water intake; (B) urine output; (C) urine osmolality; Immunoblot profiles showing the protein abundances of AQP2, AQP1 and β-actin in the inner medulla (D), cortex (E) and outer medulla (F). (G) mean densities of AQP2 protein bands relative to β-actin in the inner medulla and cortex of clopidogrel-treated group plotted as percent of the respective values in the control group. N = 4 rats/group. **P* < 0.03 and ***P* < 0.01 vs. control group by unpaired *t* test.

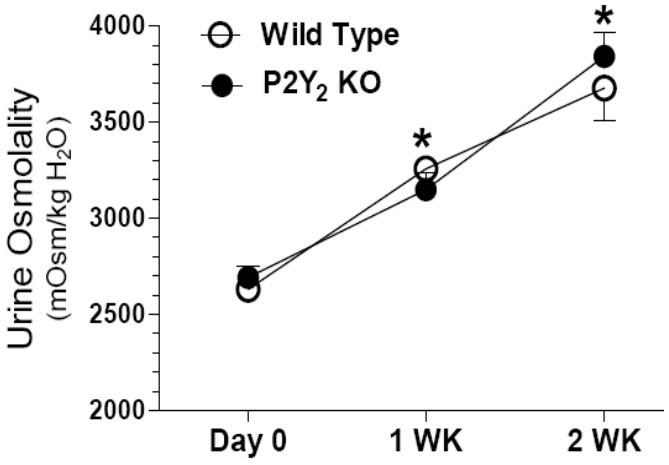

Fig. S6. Lack of interaction of clopidogrel with $P2Y_2$ receptor. $P2Y_2$ receptor knockout (KO) and wild type mice (N = 7 mice/genotype) were administered clopidogrel (80 mg/kg bw/day) in drinking water for 2 weeks (WK). Twenty-four hour urine osmolalities were monitored on days 0, and at the end of the first and second weeks. *significantly different from the corresponding day 0 values.

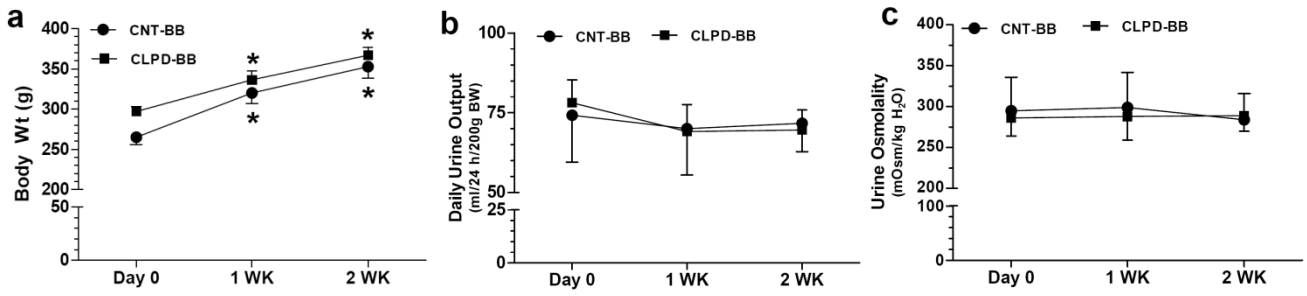

Fig. S7: **Lack of effect of clopidogrel in Brattleboro rats.** Brattleboro rats (BB) were administered clopidogrel (CLPD; 40 mg/kg bw/day) in drinking water for 2 weeks (WK). Control rats (CNT) did not receive clopidogrel. Body weights, and twenty-four hour urine osmolalities were monitored on days 0, and at the end of the first and second weeks. N = 4 rats per group.