SUPPLEMENTAL MATERIAL

1. Methods

1.1. FluxOR thallium assay

The CHO-KCNQ2 cells were seeded in wells of 96-well plates at the density of 10,000 cells/well and grown until 80 - 90% confluence, typically overnight, at 37 ℃ in a 5% CO_2 incubator. The medium was removed and 80 μl of FluxOR loading buffer (Life Technology) was added to each well for 90 min at the room temperature (22–25 ℃) in the dark. After removing the loading buffer, 100 μl assay buffer (a physiological Hank's balanced salt solution) and 20 μl 7× control/test compound, diluted in the assay buffer, were added to each well and the plate was kept in the dark for 30 min. The blank assay buffer and 100 μM ztz240 were used to represent EC_0 and EC_{100}, respectively. The plate was read using Functional Drug Screening System μ-cell FDSS (Hamamatsu, Japan) with excitation at 480 nm and emission at 540 nm at the frequency of 1-Hz. After reading for 10 s to established the baseline, 20 μl/well of a stimulus buffer containing 1.30 mM K_2SO4 and 9.80 mM Tl_2SO4 was added. The fluorescence responses from cell plates were continuously monitored for 110 s at 1-Hz frequency. The FluxOR thallium assay protocol above is identical to the manufacture's protocol. All assays were carried out at the room temperature.

1.2. Bradykinin (BK)-induced inflammatory model

Male KM mouse received an intraplantar injection of bradykinin (200μg,10μl/site) in the left hindpaws as previously described [1]. The BK-induced nocifensive responses were assessed with

score and lifting/licking time. The score was a sum of weighted pain behaviors: 1 = flinching or shaking, 2 = licking or biting. BBR (50 mg/Kg) or equivalent vehicle was intraperitoneally administered 30 min prior to the behavior test. 5 minutes after intraplantar injection of bradykinin, the responses of mice were observed and measured within 30 minutes.

1.3. Maximal electroshock (MES)-induced seizure model

Male KM mice were tested in the MES test using a physiological and pharmacological electronic stimulator (Jinan, Shandong, China). The shock level was set at 160 V and the duration was set at 5.4 s with continuous wave under the eighth configuration. The electric shock was administered using ear clip electrodes, mice with extension of hind limb were prepared for the compound tests a day later. BBR at doses of 30 and 50 mg/kg was intraperitoneally administered 30 min before electroshock application, and RTG at doses of 3, 10, 30, 60 mg/kg (p.o.) was tested in a parallel study.

1.4. Protein binding assay

To evaluate protein binding fraction of BBR in rat and mouse brain tissues, a 96-well equilibrium dialysis method was used. Briefly, fresh Sprague-Dawley rat and CD-1 mouse brain tissue were obtained on the day of the study. Brain tissue was homogenized in 3 volumes (w/v) of 50 mM sodium phosphate buffer (PBS) and the homogenate was spiked with a final test or reference compounds concentration of 1 μM containing 0.2% DMSO. With 100 μl blank dialysis buffer added to the receiver side and 100 μl brain homogenate treated with either the test or reference compound added to the donor side of the dialysis chambers, the 96-well equilibrium

dialysis apparatus was placed on a shaking device (60 rpm) in an incubator at 37°C for 5 h. At the end of the incubation, 25 µL aliquots were taken from both the donor sides and receiver sides of the dialysis apparatus, mixed with same volume of PBS and brain homogenate to obtain identical matrix. The mixtures were then quenched with 150 µL acetonitrile containing internal standard. The samples were then vortexed, centrifuged, and the supernatant was stored at -20 °C until LC/MS/MS analysis. To determine $[Initial]_{0h}$, aliquots 25 µL of the brain tissue spiked with test or reference compounds diluted in 25 µL PBS and quenched with 150 µL acetonitrile containing internal standard.

1.5. Data analysis

For FluxOR thallium assay, the ratios of fluorescence (F/F_0) at 35 s were corrected for background autofluorscence and were used for quantification of KCNQ2 channel responses to the compound using the formula: $F/F_0 = ((R_{test} - R_{control}) / (R_{control} - R_{buffer}) \times 100\%)$. The fraction of compound bound was calculated by the formula: $Bound\% = 100 \times ([Donor]_{5h} - [Receiver]_{5h}) / [Donor]_{5h}$. Except for the data from thallium assay, protein binding assay and MES test, all data are presented as means ± sem and the significance was estimated two-tailed Student's t tests. $p \leq 0.05$ was considered significance in all cases.

2. Figures

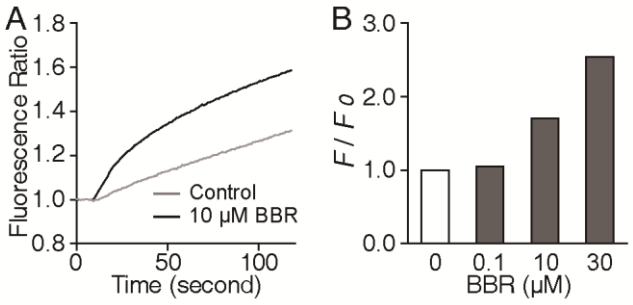

Fig. 1. Potentiation effects of BBR on KCNQ2 channel in thallium assay. (A) Representative traces of fluorescence ratio in the absence (grey) and presence of 10 μM BBR (black). (B) Dose-dependent potentiation of BBR on fluorescence signal. F_0 and F indicates fluorescence before and after BBR addition, respectively.

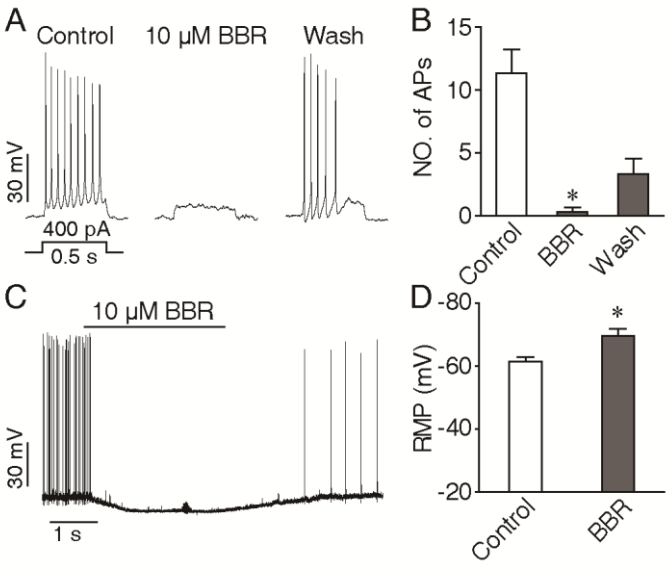

Fig. 2. BBR suppresses neuronal excitability of cultured hippocampus neurons. (A) BBR effectively suppresses the action potential firing elicited by a 400 pA current injection. (B) Bar graph showing the averaged numbers of elicited action potential spikes before, during and after removal of BBR (n = 5, *p ≤ 0.05). (C) BBR suppresses the spontaneous firing and hyperpolarizes the resting membrane potential (RMP) of hippocampus neurons. (D) Bar graph showing the hyperpolarization of RMP induced by 10 μM BBR. n = 3, *$p \leq 0.05$.

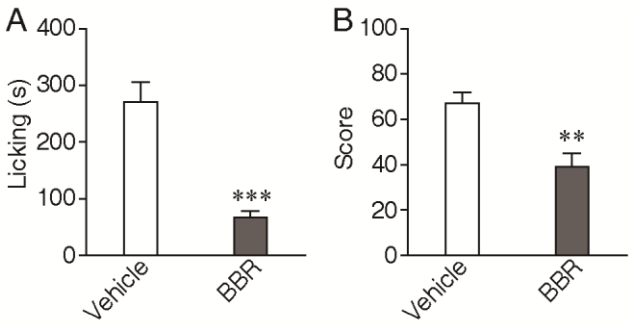

Fig. 3. BBR attenuates bradykinin (BK)-induced nociceptive behavior in mice. BBR (50 mg/Kg, i.p.) significantly suppresses intraplantar injected bradykinin (200 μg, 10 μl/site) induced licking behavior (A) and score (B). BBR was intraperitoneally administered 30 min prior to the behavior test. n = 12, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

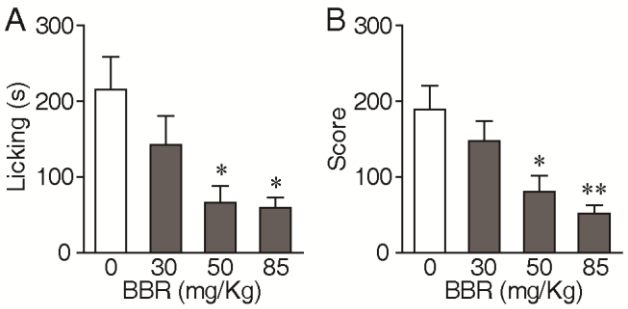

Fig. 4. BBR dose dependently relieves formalin-induced behaviors in mice. BBR attenuates both the licking time (A) and score (B) in a dose-dependent manner. Formalin (4% concentration, 30 μl) was intraplantarly injected into the right hind paw 30 min after the intraperitoneal administration of different concentration of BBR as indicated. n = 6 - 12, $*p \leq 0.05$, $**p \leq 0.01$.

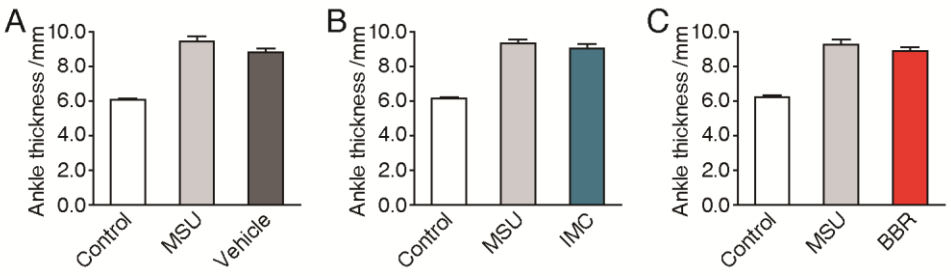

Fig. 5. Effects of intraperitoneal administration of IMC and BBR on the edema induced by intra-articular injection of MSU crystals (1.25 mg/site). Compared with the ankle thickness before (Control) and 20 h after MSU injection (MSU), needle-shaped MSU crystals induced significantly edema in the ankle. However, i.p.of vehicle (**A**), 10 mg/Kg IMC (**B**) or 50 mg/Kg BBR (**C**) had no significant effect on the edema measured 3h after administration. n = 6, ***$p \leq$ 0.001.

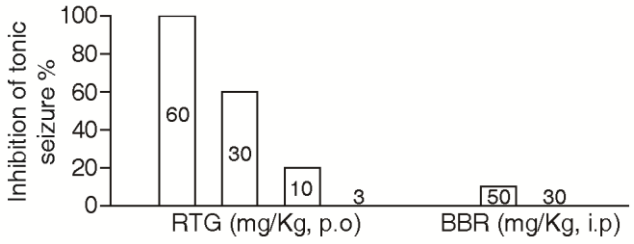

Fig. 6. Effects of retigabine (RTG) and BBR on MES-induced seizure in mice. RTG and BBR were administered 30 min prior to the MES test. The dose of drugs were given in the bar. n = 12 - 15.

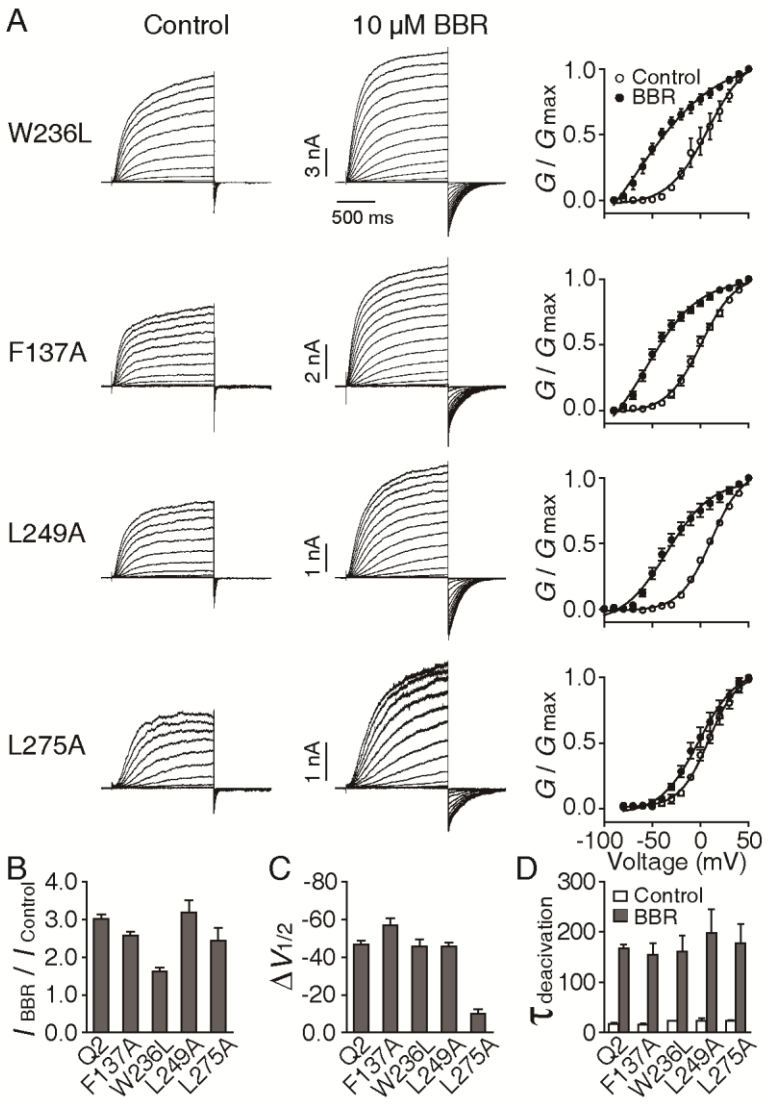

Fig. 7. Molecular determinants of BBR are distinct from previously identified residues for RTG (W236L), ztz240 (F137A) and ZnPy (L249A and L275A). (A) Representative traces and voltage-dependent activation curves of KCNQ2 mutants in the absence and presence of 10 μM BBR. The cells transfected with KCNQ2 mutants were held at -100 mV and depolarized from -90 mV to +50 mV in 10-mV increments, followed by a hyperpolarizing step to -120 mV. (B to D) Bar graphs showing the effects of 10 μM BBR on the steady-state current amplitudes recorded at -10 mV (B), left-shift of $V_{1/2}$ (C) and the deactivation time constants (D). The tale currents were

measured at -120 mV after the depolarization to +50 mV and fitted by a single exponential function. $n \geq 4$.

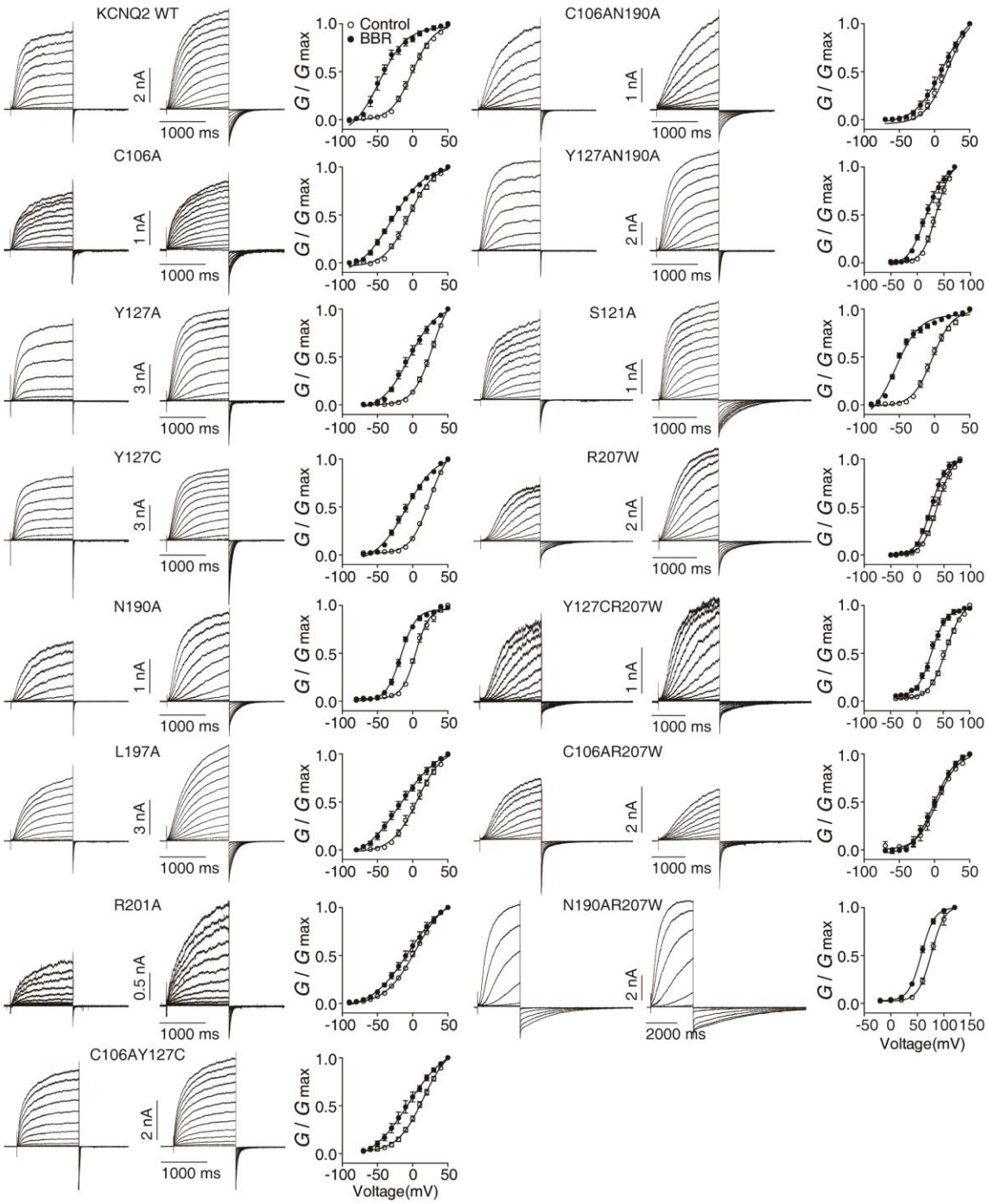

Fig. 8. The representative traces and voltage-dependent activation curves of KCNQ2 WT and mutants in the presence and absence of 10 μM BBR. The currents were elicited by a series of depolarization from -90 mV to +50 mV with a 10 mV increments from the holding potential -100 mV. n > 3 for each channel.

3. **Tables**

Table 1

Effects of RTG and FLP on the MSU-induced pain behaviors.

Compound	Animal NO.	Paw pressure score			
		MSU [a]	Time after administration [b]		
			1 h	2 h	3 h
	1	2	1	1	1.5
RTG	2	2.5	2	2.5	2.5
i.p.	3	3	3	3	3
12.5 mg/Kg	4	2.5	1.5	0.5	2
	5	2	1	0.5	2
	1	2	2	1	2
FLP	2	3	2.5	2.5	2
i.p.	3	2.5	1	1	2.5
20 mg/Kg	4	2.5	1.5	1	1.5
	5	3	1	3	2
	6	3	3	3	2.5

[a] MSU represents the paw pressure score observed 20 h after intra-articular injection of 1.25 mg/site needle-shaped MSU crystals using the methods description in the Materials and Methods.

[b] Time after administration represents the time course of paw pressure score after intraperitoneal

administration of the compounds as indicated.

Table 2

Brain protein binding of BBR.

Compound	Species	Bound [a] (%)	Fu [b] (%)	Recovery [c] (%)	Category[d]
BBR	rat	100.00	0.00	101.23	High
	mouse	100.00	0.00	99.59	High
Warfarin	rat	38.45	61.55	106.76	Moderate
	mouse	44.81	55.19	107.55	Moderate
Quinidine	rat	84.07	15.93	101.11	High
	mouse	88.51	11.49	100.76	High

[a] Bound % = 100 × ($[Donor]_{5h}$ - $[Receiver]_{5h}$) / $[Donor]_{5h}$

[b] Fu % = 100 – Bound %

Fu: Unbound Fraction

[c] Recovery % = 100 × ($[Donor]_{5h}$ + $[Receiver]_{5h}$) / $[Initial]_{0h}$

[d] Category: According to the bound fraction, compounds could be separated into three categories i.e. high, moderate and low. The bound fraction values between 50-90% in brain suggested moderate binding and above 90% belong to high binding affinity.

All values are means of duplicate results.

Table 3

Effects of BBR on KCNQ2 VSD mutants.

	Mutants	I / I_0 [a]	$\Delta V_{1/2}$ [b] (mV)	Tau [c] Control (ms)	Tau [c] + BBR (ms)
	Q2 wt	3.02±0.12	-46.56±4.50	17.50±1.69	167.60±7.47
S1	C106A	1.21±0.04	-27.79±5.16	34.64±5.12	155.17±23.04
	L107A	9.28±1.65	-40.66±6.41	8.85±0.76	81.17±6.52
	V108A	NA	NA	NA	NA
	L109A	3.27±0.36	NA	NA	NA
	S110A	NA	NA	NA	NA
	V111A	1.83±0.23	-40.91±4.16	10.79±0.06	45.81±6.46
	F112A	10.99±1.65	-61.98±2.82	14.99±0.61	112.18±11.13
	T114A	10.44±0.83	-47.21±8.07	14.99±1.64	72.99±6.09
	I115A	5.51±0.68	-43.07±3.44	13.18±1.16	76.14±6.39
S1-S2	E117A	NA	NA	NA	NA
loop	K120A	3.28±0.24	-49.01±4.22	18.61±1.12	155.51±14.08
	S121A	2.12±0.19	-52.22±3.20	13.96±0.86	203.40±23.53
	L126A	2.13±0.13	-40.28±3.83	17.95±2.90	111.57±11.63
S2	Y127A	8.84±1.18	-33.30±3.67	8.89±0.55	31.41±2.56
	Y127C	7.29±0.70	-33.61±3.67	13.06±0.98	59.47±4.65

Table 3, Continued

S2	E130A	4.95±0.80	-38.35±2.89	12.99±0.64	51.18±5.11
	F137A	2.58±0.10	-56.84±7.23	16.49±2.05	155.31±21.50
S3	G186A	2.17±0.14	-33.50±4.59	18.37±2.20	64.10±6.69
	S187A	3.47±0.42	-51.07±4.69	14.58±2.33	95.65±12.53
S3-S4	Q188A	2.59±0.39	-50.34±6.86	21.77±4.83	259.31±40.66
loop	G189A	4.07±0.87	-46.49±2.96	11.40±0.53	89.76±11.21
	N190A	3.13±0.20	-20.59±1.55	22.00±3.03	209.66±48.45
	A193V	2.73±0.41	-60.03±4.74	10.78±1.48	111.63±25.65
	T194A	1.52±0.14	-42.15±12.81	29.14±4.69	254.74±22.61
	S195A	7.60±0.51	-50.34±4.20	12.92±1.81	113.48±6.79
	A196V	2.91±0.11	-54.83±5.92	12.08±0.95	104.90±5.48
S4	L197A	2.94±0.39	-25.93±3.63	23.42±3.72	104.61±11.38
	R198A	1.08±0.06	-0.12±1.89	51.32±5.13	56.25±5.99
	L200A	7.83±1.05	-32.14±4.83	9.40±0.27	37.59±3.16
	L200W	1.84±0.11	-34.80±4.11	30.56±1.52	183.01±13.55
	R201A	5.56±0.90	-29.78±3.94	21.61±3.19	46.90±4.45
	R207W	3.01±0.48	-19.23±4.46	42.23±12.37 556.47±73.28	63.89±7.82 529.92±49.50

Table 3, Continued

C106AR207W	0.75±0.06	-3.67±1.19	108.01±31.15 445.06±79.63	191.00±57.93 725.88±76.08
C106AN190A	1.55±0.20	-6.12±2.66	50.18±2.30	268.32±23.71
C106AY127C	2.30±0.15	-21.16±3.13	8.48±0.64	31.71±3.29
Y127AN190A	4.85±0.37	-19.82±1.62	10.88±0.54	40.96±5.91
Y127CR207W	1.29±0.11	-27.48±1.18	67.67±5.23 455.32±48.23	58.84±6.43 659.81±73.20
N190AR207W	3.27±0.44	-18.57±1.38	325.72±65.65 1466.04±210.21	210.72±58.92 1943.15±350.98

[a] I / I_0 represents the effect of 10 μM BBR on outward current of the KCNQ2 wild type (wt) or mutant channels. I_0: the outward current amplitude in the absence of BBR. I: the outward current amplitude in the presence of 10 μM. For the mutants G189A and Y127AN190A, the outward currents were meausured at +10 mV; for Y127CR207W and N190AR207W, the outward currents were measured at +50 mV; for all the rest mutants, the outward currents were measured at -10 mV.

[b] $\Delta V_{1/2}$ represents the changing of $V_{1/2}$ with and without 10 μM BBR. To calculate $V_{1/2}$, the tail current elicited following -120 mV was measured.

[c] Tau Control and +BBR: time constants of mutants in the absence and presence of 10 μM BBR. The tale currents were measured at -120 mV after the depolarization to +50 mV and fitted by a

single or bi-exponential equation.

n > 3 for each mutant. The values are presented as mean ± sem.

4. References

[1] Liu B, Linley JE, Du X, Zhang X, Ooi L, Zhang H, Gamper N. The acute nociceptive signals induced by bradykinin in rat sensory neurons are mediated by inhibition of M-type K^+ channels and activation of Ca^{2+}-activated Cl^- channels. J Clin Invest 2010;120:1240-1252.