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Supplementary Materials for

Identification of GPR83 as the receptor for the neuroendocrine peptide PEN

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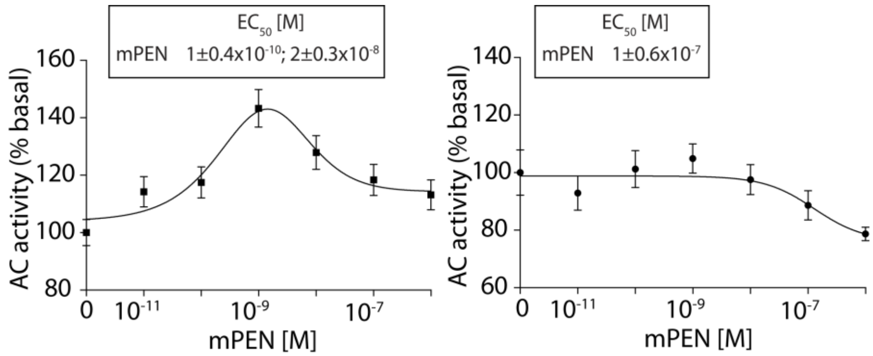

fig. S1. Variable results with studies examining the effect of mPEN on adenylyl cyclase activity in hypothalamic membranes. The effect of mPEN on adenylyl cyclase (AC) activity in mouse hypothalamic membranes (2 μg). (**Left Panel**) Data represent mean ± SE of 6 individual experiments in triplicate. (**Right Panel**) Data represent mean ± SE of 4 individual experiments in triplicate.

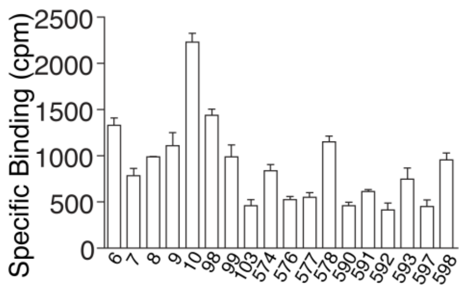

fig. S2. The binding of mPEN to hypothalamic membranes from individual male mice. The relative amount of [^{125}I]Tyr-rPEN binding in 18 individual hypothalami was examined in triplicate. The number on the x-axis denotes the individual animal number.

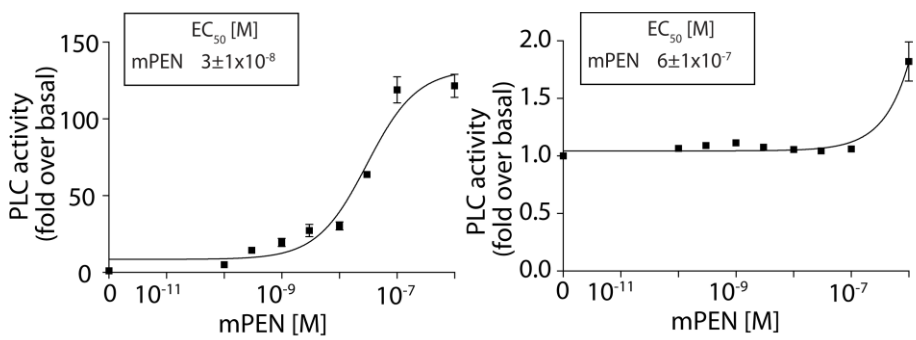

fig. S3. Variable results with studies examining the effect of mPEN on PLC activity in hippocampal membranes. The effect of mPEN on PLC activity in mouse hippocampal membranes (10 μg). (**Left Panel**) Data represent mean ± SE of 3 individual experiments in triplicate. (**Right Panel**) Data represent mean ± SE of 4 individual experiments in triplicate.

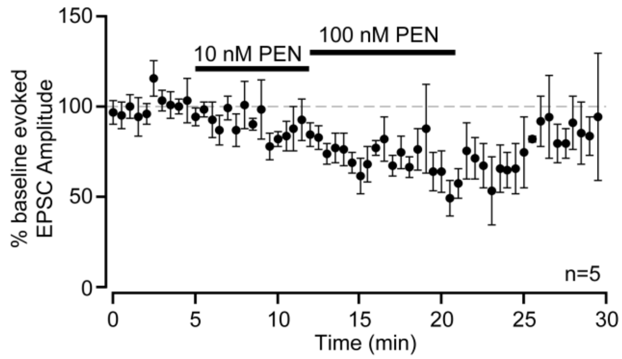

fig. S4. Evoked EPSC amplitude after sequential application of increasing concentrations of mPEN and washout. The time course of evoked EPSC amplitude following consecutive application of 10 nM and 100 nM of PEN in five PVN neurons from a single rat. Wash out of PEN effects following 5 minutes after removal of peptide from the perfusion bath. Data represent mean ± SE (n=5 individual neurons).

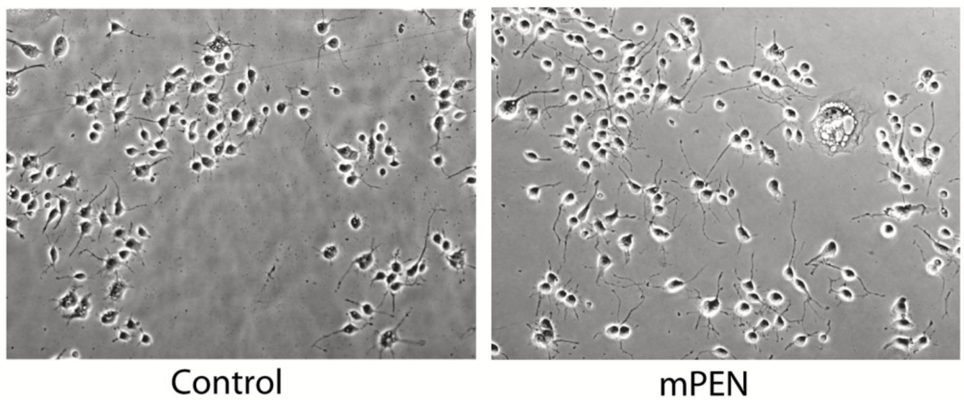

fig. S5. mPEN-stimulated neurite outgrowth in Neuro2A cells. Cells treated without or with mPEN were imaged at 20X and scored for neurite outgrowth. Cells containing neurites that were longer than twice the diameter of the cell body were scored as positive. Data from multiple images were analyzed for the graphs shown in Figures 3A and 5E.

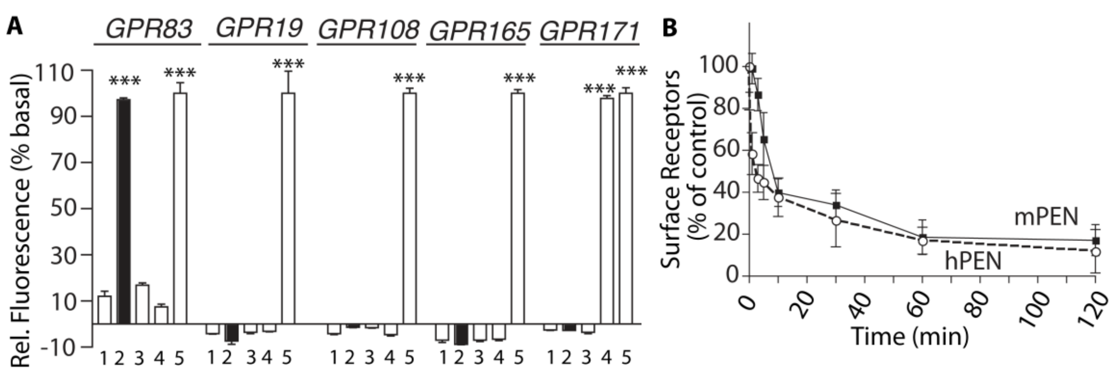

fig. S6. Expression of *GPR83* in heterologous cells confers PEN signaling and receptor endocytosis. (**A**) The effect of PEN (1 μM) on intracellular Ca^{+2} release in cells expressing *GPR83* along with a promiscuous chimeric $hG\alpha_{16/i3}$ protein. (Bar 1) scrambled peptide (1 μM), (Bar 2) hPEN (1 μM), (Bar 3) rlittleLEN (1 μM), (Bar 4) mbigLEN (1 μM) or (Bar 5) ATP (1 μM). Similar experiments were performed with mGPR19, mGPR108, mGPR165, and mGPR171. (**B**) The effect of mPEN or hPEN (100 nM) on internalization of GPR83 in HEK293 cells expressing mGPR83 (2 x 10^5 cells). Data represent mean± SE (n=3-8 independent experiments). ***p<0.001(one-way Anova for **A**); details of the statistical analyses are in table S1.

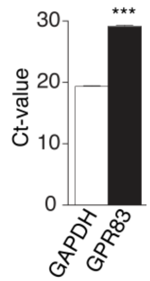

fig. S7 Quantitative RT-PCR to confirm the presence of *GPR83* mRNA in Neuro2A cells. n=6 different samples.

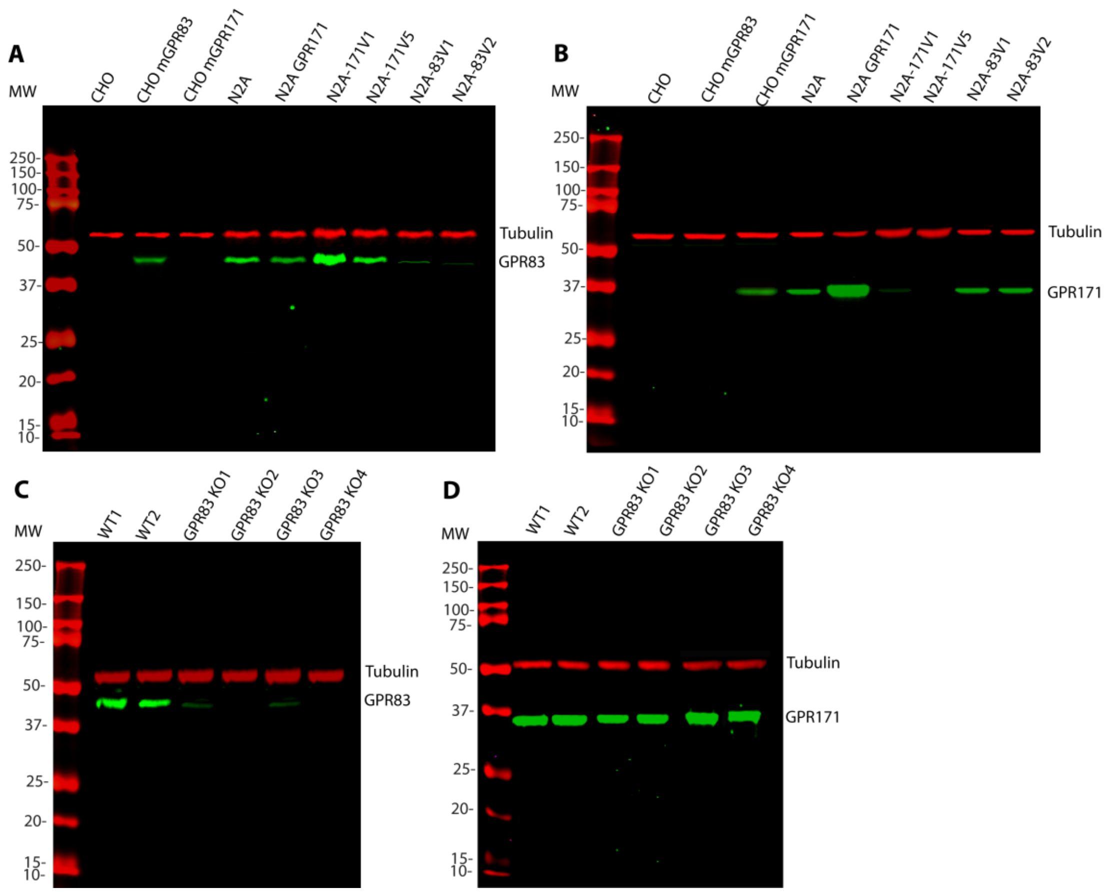

fig. S8. Specificity of the GPR83 and GPR171 antibodies. (**A**) Western blot for GPR83 in lysates (~20-35 μg protein) of CHO cells alone (CHO) or expressing either HA-tagged mGPR83 or myc-tagged mGPR171*,* of Neuro2A cells alone (N2A), Neuro2A cells overexpressing myc-tagged mGPR171 (N2A GPR171), Neuro2A cells stably expressing lentiviral shRNA to GPR171 (N2A-171V1 and N2A-171V5 generated as described in (13)), Neuro2A cells stably expressing lentiviral shRNA to GPR83 (N2A-83V1 for shRNA clone ID:TRCN0000026837, and N2A-83V2 for shRNA clone ID:TRCN0000026857) using GPR83 antibody raised in rabbit (green; 1:1000). Antibodies recognizing tubulin raised in mouse (red; 1:50,000) were used as loading control. (**B**) A parallel blot was probed with GPR171 antibody raised in rat (green; 1:5000) and antibodies recognizing tubulin (red; 1:50,000). (**C**) Western blot for GPR83 in lysates (~20-35 μg protein) of hypothalamic membranes from two individual wild type and four individual *GPR83*-knockout (KO) mice. GPR83 antibody raised in rabbit (green; 1:1000); antibodies recognizing tubulin raised in mouse (red; 1:50,000). (**D**) A parallel blot was probed with GPR171 antibody raised in rat (green; 1:5000) and antibodies recognizing tubulin (red; 1:50,000).

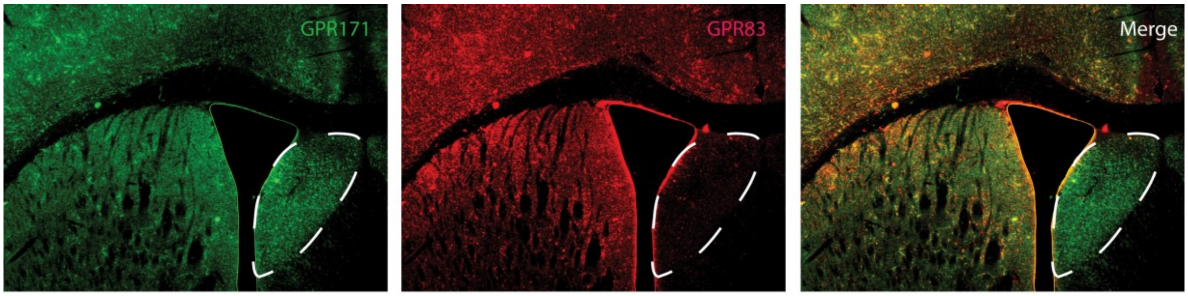

fig. S9. **Confirmation of antibody specificity and accuracy of colocalization by analysis of the lateral septum**. Immunohistochemical localization using a GPR83 antibody raised in rabbit (green) and a GPR171 antibody raised in rat (red) in the lateral septum (white dashed line) to confirm specificity of colocalization.

table S1. Description of statistical analysis for different figures. ATP, adenosine triphosphate; Cont, control; k0, knockout; n.a., not applicable; Scr. pep., scrambled peptide; WT, wild type; Dfn, degrees of freedom (df) in the numerator; Dfd, degrees of freedom (df) in the denominator.

Fig.	**t-test**		**One-way ANOVA**		**Two–way ANOVA**		
	Group	**p-value**	**Test**	**p-value**	**Test**	**p-value**	**F (DFn, DFd)**
2B	Baseline vs PEN	p<0.05; t=-5.41; df=7	n.a.	n.a.	n.a.	n.a.	n.a.
2D	Baseline vs PEN	p<0.05; -2.49; df=7	n.a.	n.a.	n.a.	n.a.	n.a.
3A	Cont v/s mPEN Cont v/s bigLEN Cont v/s SAAS mPEN v/s bigLEN mPEN v/s SAAS bigLEN v/s SAAS	p<0.0001; t=69.3; df=4 p<0.0001; t=36.3;df=8.9 p<0.0001; t=38; df=3.2 p<0.0001; t=11.8;df=8.9 p<0.0012; t=11; df=3.2 p<0.0001; t=16.3;df=9.9	**One-Way ANOVA** **Post-hoc Dunnett's test** Cont v/s mPEN Cont v/s bigLEN Cont v/s SAAS **Tukey's Multiple Comparison** Cont v/s mPEN Cont v/s bigLEN Cont v/s SAAS mPEN v/s bigLEN mPEN v/s SAAS bigLEN v/s SAAS	**p<0.0001; F=209.9** p<0.0001; df=14 p<0.0001; df=14 p<0.0001; df=14 p<0.0001; df=14 p<0.0001; df=14 p<0.0001; df=14 p<0.0001; df=14 p<0.04; df=14 p<0.0001; df=14	n.a.	n.a.	n.a.
3H	5'Cont v/s 5'mPEN 30'Cont v/s 30'mPEN	p=0.005; t=4.77; df=5 p=0.73; t=0.365; df=5.03	n.a.	n.a.	Interaction Treatment Time **Tukey's Multiple Comparison** 5'Cont v/s 5'mPEN 5'Cont v/s 30'Cont 5'Cont v/s 30'mPEN 5'mPEN v/s 30'Cont 5'mPEN v/s 30'mPEN 30'Cont v/s 30'mPEN	p=0.0002 p<0.0001 p=0.0002 p<0.0001 p>0.9999 p=0.9982 p<0.0001 p<0.0001 p=0.9982	F (1, 20) = 21.53 F (1, 20) = 23.97 F (1, 20) = 21.53 df=20 df=20 df=20 df=20 df=20 df=20
4A and S6A	n.a.	n.a.	**GPR83** **One-way ANOVA** **Dunnett's test** Scr.pep v/s hPEN Scr.pep v/s littleLEN	**p<0.0001; F=216.2** p<0.0001; df=42	n.a.	n.a.	n.a.

			Scr.pep v/s bigLEN Scr.pep v/s ATP **GPR19** **One-way ANOVA** **Dunnett's test** Scr.pep v/s hPEN Scr.pep v/s littleLEN Scr.pep v/s bigLEN Scr.pep v/s ATP **GPR108** **One-way ANOVA** **Dunnett's test** Scr.pep v/s hPEN Scr.pep v/s littleLEN Scr.pep v/s bigLEN Scr.pep v/s ATP **GPR165** **One-way ANOVA** **Dunnett's test** Scr.pep v/s hPEN Scr.pep v/s littleLEN Scr.pep v/s bigLEN Scr.pep v/s ATP **GPR171** **One-way ANOVA** **Dunnett's test** Scr.pep v/s hPEN Scr.pep v/s littleLEN Scr.pep v/s bigLEN Scr.pep v/s ATP	p=0.9997; df=42 p=0.8748; df=42 p<0.0001; df=42 **p<0.0001;** **F=111.4** p=0.9755; df=18 p=0.9999; df=18 p=0.9997; df=18 p<0.0001; df=18 **p<0.0001;** **F=2233** p=0.0832; df=17 p=0.1219; df=17 p=0.9945; df=17 p<0.0001; df=17 **p<0.0001;** **F=2505** p=0.5171; df=18 p=0.9999; df=18 p=0.9962; df=18 p<0.0001; df=18 **p<0.0001;** **F=2019** p>0.9999; df=18 p=0.9667; df=18 p<0.0001; df=18 p<0.0001; df=18			
4F	-PTX v/s + PTX	p=0.0005; t=4.307; df=16	n.a.	n.a.	n.a.	n.a.	n.a.
5A	50 pmol cont v/s 50 pmol GPR83 100 pmol cont v/s 100 pmol GPR83 200 pmol cont v/s 200 pmol GPR83	p<0.0001;t=30.82; df=4 p<0.0001;t=34.62; df=4 p<0.0001;t=97.07; df=4	n.a.	n.a.	Interaction SiRNA type Dose **Tukey's Multiple Comparison** 50 pmol cont v/s 50 pmol GPR83 50 pmol cont v/s 100 pmol cont 50 pmol cont v/s 100 pmol GPR83 50 pmol cont v/s 200 pmol cont 50 pmol cont v/s	p<0.0001 p<0.0001 p<0.0001 p<0.0001 p>0.9999 p<0.00	F (2, 12) = 67.82 F (1, 12) = 5458 F (2,12) = 67.82 df=12 df=12 df=12 df=12

					200 pmol GPR83 50 pmol GPR83 v/s 100 pmol cont 50 pmol GPR83 v/s 100 pmol GPR83 50 pmol GPR83 v/s 200 pmol cont 50 pmol GPR83 v/s 200 pmol GPR83 100 pmol cont v/s 100 pmol GPR83 100 pmol cont v/s 200 pmol cont 100 pmol cont v/s 200 pmol GPR83 100 pmol GPR83 v/s 200 pmol cont 100 pmol GPR83 v/s 200 pmol GPR83 200 pmol cont v/s 200 pmol GPR83	01 p>0.9999 p<0.0001 p<0.0001 p<0.0016 p<0.0001 p<0.0001 p<0.0001 p>0.9999 p<0.0001 p<0.0001 p<0.0001 p<0.0001	df=12 df=12 df=12 df=12 df=12 df=12 df=12 df=12 df=12 df=12 df=12
5B	Cont v/s GPR83 siRNA	p<0.0001;t=8.84; df=6.941	n.a.	n.a.	n.a.	n.a.	n.a.
5C	Cont siRNA (-PEN) v/s (+PEN) GPR83 siRNA (-PEN) v/s (+PEN)	p<0.0001;t=8.35; df=9.983 p=0.114;t=1.74; df=9.47	n.a.	n.a.	Interaction SiRNA type PEN conc. **Tukey's Multiple Comparison** Cont siRNA (-PEN) v/s (+PEN) Cont siRNA (-PEN) v/s GPR83 siRNA(-PEN) Cont siRNA (-PEN) v/s GPR83 siRNA(+PEN) Cont siRNA (+PEN) v/s GPR83 siRNA(-PEN) Cont siRNA (+PEN) v/s GPR83 siRNA(+PEN)	p=0.0061 p=0.0061 p<0.0001 p<0.0001 p>0.9999 p=0.1963 p<0.0001	F (1, 20) = 9.42 F (1, 20) = 9.42 F (1, 20) = 35.98 df=20 df=20 df=20 df=20 df=20

					GPR83 siRNA(-PEN) v/s GPR83 siRNA(+PEN)	p<0.0017 p=0.1963	df=20
5D	Cont siRNA (-PEN) v/s (+PEN) GPR83 siRNA (-PEN) v/s (+PEN)	p<0.005; t=4.77; df=5 p<0.0001; t=31.07; df=5			Interaction SiRNA type PEN conc. **Tukey's Multiple Comparison** Cont siRNA (-PEN) v/s (+PEN) Cont siRNA (-PEN) v/s GPR83 siRNA(-PEN) Cont siRNA (-PEN) v/s GPR83 siRNA(+PEN) Cont siRNA (+PEN) v/s GPR83 siRNA(-PEN) Cont siRNA (+PEN) v/s GPR83 siRNA(+PEN) GPR83 siRNA(-PEN) v/s GPR83 siRNA(+PEN)	p<0.0001 p<0.0001 p<0.0022 p<0.0001 p>0.9999 p=0.3083 p<0.0001 p<0.0001 p=0.3083	F (1, 20) = 36.34 F (1, 20) = 36.34 F (1, 20) = 12.26 df=20 df=20 df=20 df=20 df=20 df=20
5E	Cont siRNA (-PEN) v/s (+PEN) GPR83 siRNA (-PEN) v/s (+PEN)	p<0.0001; t=69.3; df=10 p<0.3466; t=1; df=8			Interaction SiRNA type PEN conc. **Tukey's Multiple Comparison** Cont siRNA (-PEN) v/s (+PEN) Cont siRNA (-PEN) v/s GPR83 siRNA(-PEN) Cont siRNA (-PEN) v/s GPR83 siRNA(+PEN) Cont siRNA (+PEN) v/s GPR83 siRNA(-PEN) Cont siRNA (+PEN) v/s GPR83 siRNA(+PEN)	p<0.0001 p<0.0001 p<0.0001 p<0.0001 p>0.0033 p>0.0417 p<0.0001	F (1, 20) = 1536 F (1, 20) = 2017 F (1, 20) = 1667 df=20 df=20 df=20 df=20 df=20

					GPR83 siRNA(-PEN) v/s GPR83 siRNA(+PEN)	p<0.0001 p<0.6611	df=20
5F	n.a.	n.a.	**One-Way ANOVA** **Tukey's Multiple Comparison** WT 574 v/sWT 576 WT 574 v/s WT 578 WT 574 v/s GPR83 ko 572 WT 574 v/s GPR83 ko 582 WT 574 v/s GPR83 ko 585 WT 576 v/s WT 578 WT 576 v/s GPR83 ko 572 WT 576 v/s GPR83 ko 582 WT 576 v/s GPR83 ko 585 WT 578 v/s GPR83 ko 582 WT 578 v/s GPR83 ko 572 WT 578 v/s GPR83 ko 585 GPR83 ko 572 v/s GPR83 ko 582 GPR83 ko 572 v/s GPR83 ko 585 GPR83 ko 582 v/s GPR83 ko 585	**p<0.0001; F=116.3** p<0.0022; df=12 p<0.0021; df=12 p<0.0001; df=12 p<0.0001; df=12 p<0.0001; df=12 p<0.0001; df=12 p=0.0002;df=12 p=0.0001;df=12 p<0.0001; df=12 p<0.0001; df=12 p<0.0001; df=12 p<0.0001; df=12 p=0.9998; df=12 p=0.9056; df=12 p=0.9694; df=12	n.a.	n.a.	n.a.
5G	WT574(-PEN) v/s WT574(+PEN) WT576(-PEN) v/s WT576(+PEN) WT578(-PEN) v/s WT578(+PEN	p<0.0001; t=6.699; df=9.89 p<0.0011; t=4.899; df=8.201 p<0.0011; t=8.357; df=9.983 p=0.0032; t=3.953; df=9.166 p=0.5360; t=0.6415; df=9.72			Interaction Mouse type PEN conc. **Tukey's Multiple Comparison** WT574(-PEN) v/s WT574(+PEN) WT574(-PEN)	p<0.0001 p<0.0001 p<0.0001 p<0.0001	F (5, 60) = 13.39 F (5, 60) = 13.51 F (1, 60) = 116.7 df=60

	) GPR83 ko 572(-PEN) v/s GPR83 ko 572(+PEN) GPR83 ko 582(-PEN) v/s GPR83 ko 582(+PEN) GPR83 ko 585(-PEN) v/s GPR83 ko 585(+PEN)	p=0.8096; t=0.2530; df=5.402			v/s WT576(-PEN) WT574(-PEN) v/s WT576(+PEN) WT574(-PEN) v/s WT578(-PEN) WT574(-PEN) v/s WT578(+PEN) WT574(-PEN) v/s GPR83 ko 572(-PEN) WT574(-PEN) v/s GPR83 ko 572 (+PEN) WT574(-PEN) v/s GPR83 ko 582(-PEN) WT574(-PEN) v/s GPR83 ko 582 (+PEN) WT574(-PEN) v/s GPR83 ko 585(-PEN) WT574(-PEN) v/s GPR83 ko 585 (+PEN) WT574(+PEN) v/s WT576(-PEN) WT574(+PEN) v/s WT576(+PEN) WT574(+PEN) v/s WT578(-PEN) WT574(+PEN) v/s WT578(+PEN) WT574(+PEN) v/s GPR83 ko 572(-PEN) WT574(+PEN) v/s GPR83 ko 572(+PEN) WT574(+PEN) v/s GPR83 ko 582(-PEN) WT574(+PEN) v/s GPR83 ko 582(+PEN) WT574(+PEN) v/s GPR83 ko 585(-PEN) WT574(+PEN) v/s GPR83 ko 585(+PEN) WT576(-PEN) v/s WT576(+PEN)	p>0.9999 p<0.0001 p>0.9999 p<0.0001 p>0.9999 p=0.1626 p>0.9999 p>0.9999 p>0.9999 p>0.9999 p<0.0001 p>0.9917 p<0.0001 p>0.9851 p<0.0001 p<0.0013 p<0.0001 p<0.00	df=60 df=60

					WT576(-PEN) v/s WT578(- PEN) WT576(-PEN) v/s WT578(+PEN) WT576(-PEN) v/s GPR83 ko 572(-PEN) WT576(-PEN) v/s GPR83 ko 572(+PEN) WT576(-PEN) v/s GPR83 ko 582(-PEN) WT576(-PEN) v/s GPR83 ko 582(+PEN) WT576(-PEN) v/s GPR83 ko 585(-PEN) WT576(-PEN) v/s GPR83 ko 585(+PEN) WT576(+PEN) v/s WT578(- PEN) WT576(+PEN) v/s WT578(+PEN) WT576(+PEN) v/s GPR83 ko 572(-PEN) WT576(+PEN) v/s GPR83 ko 572(+PEN) WT576(+PEN) v/s GPR83 ko 582(-PEN) WT576(+PEN) v/s GPR83 ko 582(+PEN) WT576(+PEN) v/s GPR83 ko 585(-PEN) WT576(+PEN) v/s GPR83 ko 585(+PEN) WT578(-PEN) v/s WT578(+PEN) WT578(-PEN) v/s GPR83 ko 572(-PEN) WT578(-PEN) v/s GPR83 ko 572(+PEN) WT578(-PEN) v/s GPR83 ko 582(-PEN) WT578(-PEN) v/s GPR83 ko	01 $p<0.00$ 01 $p<0.00$ 01 $p<0.00$ 01 $p>0.99$ 99 $p<0.00$ 01 $p>0.99$ 99 $p=0.14$ 42 $p>0.99$ 99 $p>0.99$ 99 $p>0.99$ 99 $p>0.99$ 99 $p<0.00$ 01 $p=0.45$ 01 $p<0.00$ 01 $p=0.04$ 47 $p<0.00$ 01	df=60 df=60

						582(+PEN) WT578(-PEN) v/s GPR83 ko 585(-PEN) WT578(-PEN) v/s GPR83 ko 585(+PEN) WT578(+PEN) v/s GPR83 ko 572(-PEN) WT578(+PEN) v/s GPR83 ko 572(+PEN) WT578(+PEN) v/s GPR83 ko 582(-PEN) WT578(+PEN) v/s GPR83 ko 582(+PEN) WT578(+PEN) v/s GPR83 ko 585(-PEN) WT578(+PEN) v/s GPR83 ko 585(+PEN) GPR83 ko 572(-PEN) v/s GPR83 ko 572(+PEN) GPR83 ko 572(-PEN) v/s GPR83 ko 582(-PEN) GPR83 ko 572(-PEN) v/s GPR83 ko 582(+PEN) GPR83 ko 572(-PEN) v/s GPR83 ko 585(-PEN) GPR83 ko 572(-PEN) v/s GPR83 ko 585(+PEN) GPR83 ko 572(+PEN) v/s GPR83 ko 582(-PEN) GPR83 ko 572(+PEN) v/s GPR83 ko 582(+PEN) GPR83 ko 572(+PEN) v/s GPR85 ko 582(-PEN) GPR83 ko 572(+PEN) v/s GPR83 ko 585(+PEN) GPR83 ko 582(-PEN) v/s GPR83 ko 582(+PEN) GPR83 ko 582(-PEN) v/s GPR83 ko 585(-PEN)	p<0.0001 p<0.0001 p<0.0001 p<0.0001 p>0.9999 p=0.1419 p>0.9999 p>0.9999 p>0.9999 p>0.9999 p<0.0001 p<0.0001 p<0.0001 p<0.0001 p<0.0001 p<0.0001	df=60 df=60 df=60 df=60 df=60 df=60 df=60 df=60 df=60 df=60 df=60 df=60 df=60 df=60 df=60 df=60 df=60 df=60 df=60

					GPR83 ko 582(-PEN) v/s GPR83 ko 585(+PEN) GPR83 ko 582(+PEN) v/s GPR83 ko 585(-PEN) GPR83 ko 582(+PEN) v/s GPR83 ko 585(+PEN) GPR83 ko 585(-PEN) v/s GPR83 ko 585(+PEN)	p=0.1396 p>0.9999 p>0.9999 p>0.9999 p>0.9999 p=0.1440 p=0.4728 p=0.1434 p=0.1980 p>0.9999 p>0.9999 p>0.9999 p>0.9999 p>0.9999		

						p>0.99 99	
5H	WT (-PEN) v/s WT (+PEN) GPR83 ko (-PEN) v/s GPR83 ko(+PEN)	p<0.0372; t=1.969; df=11.06 p=0.3553; t=0.3781; df=15.18			Interaction Mouse type PEN conc. **Tukey's Multiple Comparison** WT (-PEN) v/s WT (+PEN) WT (-PEN) v/s GPR83 ko (-PEN) WT (-PEN) v/s GPR83 ko (+PEN) WT (+PEN) v/s GPR83 ko (-PEN) WT (+PEN) v/s GPR83 ko (+PEN) GPR83 ko (-PEN) v/s GPR83 ko (+PEN)	p=0.09 85 p<0.05 53 p<0.24 21 p=0.04 81 p>0.99 99 p=0.99 99 p=0.04 81 p=0.06 68 p=0.99 99	F (1, 44) = 2.850 F (1, 44) = 1.401 F (1, 44) = 3.877 df=44 df=44 df=44 df=44 df=44 df=44
6B	hGPR83 v/s hGPR83+mGPR171 (t)	p=0.0092; t=4.049; df=5.156	n.a.	n.a.	n.a.	n.a.	n.a.
7B			**One-way ANOVA** **Tukey´s Multiple Comparison** GPR171+GPR83+ v/s GPR171+GPR83- GPR171+GPR83+ v/s GPR171-GPR83+ GPR171+GPR83- v/s GPR171-GPR83+	**p<0.0001; F=157.3** p < 0.0001; df= 87 p < 0.0001; df = 87 p = 0.4180 ; df = 87			