Glucocorticoids rapidly modulate $Ca_V1.2$-mediated calcium signals through Kv2.1 channel clusters in hippocampal neurons

Di Wan[1,2]†, Tongchuang Lu[1,2]†, Chenyang Li[1,2], Changlong Hu[1,2]*

1. Department of Physiology and Neurobiology, School of Life Sciences, Fudan University, Shanghai 200438, P.R.China

2. International Human Phenome Institute (Shanghai), Shanghai 200433, P.R.China

†, These authors contributed equally to the work.

***Corresponding author:** Changlong Hu

Email: clhu@fudan.edu.cn

Data Availability Statement

All data supporting the findings of this study are available within the paper and its Supplementary Information.

Conflict of Interest Statement

The authors declare no competing financial interests.

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Abstract

The precise regulation of Ca^{2+} signals plays a crucial role in the physiological functions of neurons. Here, we investigated the rapid effect of glucocorticoids on Ca^{2+} signals in hippocampal neurons. In cultured hippocampal neurons, glucocorticoids inhibited the spontaneous somatic Ca^{2+} spikes generated by Kv2.1-organized Ca^{2+} microdomains. Furthermore, glucocorticoids rapidly reduced the cell surface expressions of Kv2.1 and $Ca_V1.2$ channels in hippocampal neurons. In HEK293 cells transfected with Kv2.1 alone, glucocorticoids significantly reduced the surface expression of Kv2.1 with little effect on K^+ currents. Glucocorticoids inhibited $Ca_V1.2$ currents but had no effect on the cell surface expression of $Ca_V1.2$ in HEK293 cells transfected with $Ca_V1.2$ alone. Notably, in the presence of wild-type Kv2.1, glucocorticoids caused a decrease in the surface expression of $Ca_V1.2$ channels in HEK293 cells. However, this effect was not observed in the presence of non-clustering Kv2.1S586A mutant channels. Live cell imaging showed that glucocorticoids rapidly decreased Kv2.1 clusters on the plasma membrane. Correspondingly, western blot results indicated a significant increase in the cytoplasmic level of Kv2.1, suggesting the endocytosis of Kv2.1 clusters. Glucocorticoids rapidly decreased the intracellular cAMP concentration and the phosphorylation level of PKA in hippocampal neurons. The PKA inhibitor H89 mimicked the effect of glucocorticoids on Kv2.1, while the PKA agonist forskolin abrogated the effect. In conclusion, glucocorticoids rapidly regulate $Ca_V1.2$-mediated Ca^{2+} signals in hippocampal neurons by promoting the endocytosis of Kv2.1 channel clusters through reducing PKA activity.

Significance Statement

The rapid non-genomic effect of glucocorticoids on central nervous system is not fully understood. $Ca_V1.2$-mediated Ca^{2+} signaling microdomains control somatic Ca^{2+} signals and regulate excitation-transcription coupling in hippocampal neurons. Here, we demonstrate that glucocorticoids rapidly inhibit $Ca_V1.2$-mediated somatic Ca^{2+} spikes in hippocampal neurons. Glucocorticoids reduce the surface expression of Kv2.2 clusters and have no effect on non-clustering Kv2.2. Moreover, glucocorticoids induce the endocytosis of $Ca_V1.2$ channels through wild-type Kv2.1. However, glucocorticoids cannot induce the endocytosis of $Ca_V1.2$ channels through Kv2.1S586A mutant channels, which cannot form clusters. This study sheds light on the intricate interplay between glucocorticoids, Kv2.1 channels, and $Ca_V1.2$ channels, unraveling a dual mechanism that influences both overall Ca^{2+} signaling and the intricate organization of neural microdomains.

Introduction

Glucocorticoids, also known as stress hormones, are produced by the zona fasciculata cells of the adrenal cortex in a diurnal pattern. In the mammalian brain, glucocorticoids induce variable neuronal effects via both genomic and non-genomic signaling pathways. While the genomic pathway has been well studied, the rapid non-genomic effects of glucocorticoids, which usually occur within a few minutes, are still not fully understood. The hippocampus is the main effector of glucocorticoids in brain (1). The level of glucocorticoids shows a distinct circadian and ultradian rhythm in brain (2, 3). Recent studies have shown that the ultradian rhythmicity of glucocorticoids is critical in regulating normal emotional and cognitive responses in humans (4). Glucocorticoids may modulate neural function through rapid non-genomic effects during these fluctuations.

The Ca^{2+} entry through L-type calcium channels, mainly $Ca_V1.2$ and $Ca_V1.3$ channels, is essential for the normal functioning of various mammalian brain neurons(5, 6). Even through both mRNA expression of $Ca_V1.2$ and $Ca_V1.3$ has been suggested in the hippocampus, L-type Ca^{2+} current in hippocampal neurons may be predominantly mediated by $Ca_V1.2$ channels(7-10). Accordingly, the hippocampal pyramidal neurons in $Ca_V1.2$ knockout mice displayed a nearly complete loss of L-type Ca^{2+} currents, and $Ca_V1.2$ but not $Ca_V1.3$ knockout mice showed deficits in hippocampal long-term potentiation and memory formation(11-13).

The family of Kv2 channels, consisting of Kv2.1 and Kv2.2, plays a vital role in controlling neural excitability(14, 15). The Kv2.1 channel is widely expressed throughout the mammalian brain, and is the major conductor of rectifying potassium currents in hippocampal neurons(16, 17). Kv2.1 channels in cell membrane exist in two forms: clustered and nonclustered(18) . Nonclustered Kv2.1 channels conduct K^+ normally and are critical for maintaining repetitive firing of neurons(19, 20). Clustered Kv2.1 channels are barely conductive and serve as a platform to organize neuronal endoplasmic reticulum/plasma membrane (ER/PM) junctions(21-25). ER/PM junctions are critical for lipid and Ca^{2+} homeostasis in eukaryotic cells(26, 27),

and are particularly abundant in the soma of mammalian brain neurons(28). Recent studies have shown that clustered Kv2.1 channels recruit $Ca_V1.2$ to somatic ER/PM junctions to form Ca^{2+} signaling microdomains. These microdomains control somatic Ca^{2+} signals and regulate excitation-transcription coupling in hippocampal neurons(29, 30).

In this study, we investigated that the rapid effects of glucocorticoids on Ca^{2+} signals in hippocampal neurons. Our results show that glucocorticoids reduce cell surface expression of $Ca_V1.2$ by promoting endocytosis of clustered Kv2.1. This, in turn, inhibits somatic Ca^{2+} sparklets in hippocampal neurons through the PKA signaling pathway.

Results

Glucocorticoids reduce somatic spontaneous Ca^{2+} signals and Kv2.1 clusters in cultured hippocampal neurons.

First, we investigated the effect of glucocorticoids on somatic spontaneous Ca^{2+} signals in cultured hippocampal neurons under internal reflection fluorescence (TIRF) microscopy. The fluorescent calcium indicator Cal-590 AM was used to detect the spontaneous somatic Ca^{2+} signals in cultured hippocampal neurons. Bath application of 1 μM corticosterone for 5 min significantly reduced the frequency and amplitude of spontaneous somatic Ca^{2+} sparks in hippocampal neurons, with no effect on the spark width (Fig. 1A-C; Supplemental Video S1).

Spontaneous somatic Ca^{2+} signals in hippocampal neurons are usually generated at Kv2.1 cluster-associated ER-PM junction sites, where Kv2.1 recruits $Ca_V1.2$ to form local Ca^{2+}-release microdomains(29). Therefore, we investigated whether corticosterone regulates Ca^{2+} signaling via Kv2.1 clusters. Kv2.1 clusters with diameter more than 0.5 um were calculated (31). Treatment with 1μM corticosterone for 5 minutes reduced Kv2.1 clusters in the soma of hippocampal neurons (Fig. 1D). This suggests that corticosterone caused Kv2.1 declustering or endocytosis. To further investigate the mechanism by which glucocorticoids reduce somatic spontaneous Ca^{2+}

signals in cultured hippocampal neurons, we tested the effect of corticosterone on Ca^{2+} and K^{+} channel currents in cultured hippocampal neurons.

Glucocorticoids rapidly inhibit calcium and potassium channel currents in hippocampal neurons.

Since the predominant calcium channel currents in hippocampal neurons are conducted by L-type $Ca_V1.2$ channels (12), Ba^{2+} was used as the charge carrier to minimize Ca^{2+}-induced inactivation. Whole-cell Ba^{2+} currents in cultured hippocampal neurons were elicited by a 100-ms depolarizing pulse to 0 mV from a holding potential of -90 mV at 10 s intervals. Bath application of 1μM corticosterone significantly inhibited the Ba^{2+} currents in hippocampal neurons (Fig. 2A). The inhibitory effect of corticosterone on I_{Ba} began quickly and reached a maximum effect around 5 min (Fig. 2B). Kv2.1 channels are the major contributor of delayed rectifier potassium currents (I_k) in hippocampal neurons (16). I_k in hippocampal neurons was elicited by a 200-ms depolarizing pulse to +40 mV from a holding potential of -80 mV at 10 s intervals. 1μM corticosterone had a small inhibitory effect on I_k in hippocampal neurons (precent inhibition: 14.3 ± 2.9%, Fig. 2C). Previous studies have reported that Kv2.1 de-clustering is usually accompanied by a large hyperpolarizing shift in the steady-state activation of I_K in hippocampal neurons (32, 33). We found that extracellular application of corticosterone did not alter the steady-state activation of I_k in hippocampal neurons (Fig. 2D). This suggests that corticosterone may cause Kv2.1 cluster endocytosis instead of de-clustering. Therefore, we tested the effect of corticosterone on surface expression of Kv2.1 channels.

Glucocorticoids reduce membrane protein expressions of Kv2.1 and $Ca_V1.2$ in cultured hippocampal neurons.

Treatment of cultured hippocampal neurons with 1 μM corticosterone for a duration of 10 minutes resulted in a significant reduction in the cell surface expression of Kv2.1 channels (Fig. 2E). Additionally, the application of 1μM corticosterone also induced a substantial decrease in the cell surface expression of $Ca_V1.2$ channels in

cultured hippocampal neurons (Fig. 2F). To further explore the mechanism of glucocorticoids regulating Kv2.1 and Cav1.2 channels, we tested the inhibitory effect of glucocorticoids in heterologous HEK293 cells, which lack endogenous Kv2.1 and $Ca_V1.2$ channels(34, 35).

Glucocorticoids have little effect on Kv2.1 currents but significantly reduce the cell surface expression of Kv2.1 channels in HEK293 cells.

The primary glucocorticoids in mice and rats are corticosterone, while in humans, the primary glucocorticoids are cortisol. Therefore, we used cortisol on HEK293 cells transfected with Kv2.1 channels. Extracellular application of 1μM cortisol had little effect on Kv2.1 currents in HEK293 cells (Fig.3A). Similar to the effect of corticosterone on Kv2.1 channels in hippocampal neurons, 1μM cortisol significantly reduced the cell surface expression of Kv2.1 in HEK293 cells (Fig. 3B). Consequently, the level of Kv2.1 channel proteins in the cell cytoplasm increased (Fig. 3C). These results suggest that cortisol caused Kv2.1 endocytosis rather than de-clustering. It is well-known that clustered Kv2.1 channels are nonconducting. Since the effect of cortisol on the surface expression of the Kv2.1 channel is much greater than the effect on the current amplitude, it is reasonable to assume that the endocytosed Kv2.1 channels induced by cortisol are from non-K^+-conducting Kv2.1 clusters. Therefore, we tested the effect of cortisol on Kv2.1 clusters. We used TIRF microscopy to image the change of Kv2.1 (N-terminal GFP fusion of Kv2.1) clusters in HEK 293 cells. Consistent with previous studies (18), large GFP-Kv2.1 clusters were clearly detected by TIRF (Fig. 3D). Extracellular application of 1μM cortisol quickly reduced Kv2.1 clusters in HEK293 cells (Fig. 3D). Furthermore, we tested the effect of cortisol on mutant Kv2.1S586A channels, which lost the ability to form clusters (36). Extracellular application of 1μM cortisol for 10 minutes did not alter the surface expression of Kv2.1S586A channels (Fig. 3E). The data suggest that glucocorticoids mainly promote the endocytose of Kv2.1 clusters.

Glucocorticoids significantly inhibit $Ca_V1.2$ currents while has little effect on the

surface expression of $Ca_V1.2$ channels in HEK293 cells.

When compared to its effect on calcium channel currents in hippocampal neurons, glucocorticoids also rapidly reduced the $Ca_V1.2$ currents but with smaller amplitude in HEK293 cells (Fig. 4A). Moreover, Cortisol inhibited $Ca_V1.2$ currents at all testing potentials that more positive than -10 mV, and did not alter the steady-state activation of $Ca_V1.2$ currents (Fig. 4C). Unlike the effect on $Ca_V1.2$ in hippocampal neurons, glucocorticoids (1 μM cortisol) did not alter the cell surface expression of $Ca_V1.2$ in HEK293 cells (Fig. 4D).

Glucocorticoids reduce the cell surface expression of $Ca_V1.2$ channels when Kv2.1 and $Ca_V1.2$ are co-expressed in HEK293 cells.

Previous studies have demonstrated a spatial correlation between Kv2.1 clusters and $Ca_V1.2$ channels in cultured hippocampal neurons(29, 30). Therefore, we hypothesized that glucocorticoids affect $Ca_V1.2$ surface expression through Kv2.1 channel clusters. To test our hypothesis, we co-transfected $Ca_V1.2$ with either wild-type Kv2.1 or mutant Kv2.1S586A expression vectors in HEK293 cells. Our results showed that 1 μM cortisol significantly reduced the cell surface express of $Ca_V1.2$ channels in the presence of wild -type Kv2.1 channels in the HEK293 cells (Fig. 5A), as seen in cultured hippocampal neurons. Additionally, 1μM cortisol had a greater inhibitory effect on $Ca_V1.2$ currents in the co-transfected cells than the cells transfected with $Ca_V1.2$ alone (Fig. 5B). Furthermore, we found that 1μM cortisol did not alter the cell surface express of $Ca_V1.2$ channels in the presence of mutant Kv2.1S586A channels in HEK293 cells (Fig.5C). This suggests that glucocorticoids induce $Ca_V1.2$ channel endocytosis through Kv2.1 clusters.

Glucocorticoids regulate Kv2.1 channels through membrane-associated receptors.

Finally, we investigated how glucocorticoids regulate the membrane expression of Kv2.1 channels. The rapid effect of glucocorticoids is normally mediated by membrane-associated receptors(37). We tested the effect of cortisol-BSA conjugate

(cortisol-BSA), which cannot pass through cell membranes, on Kv2.1 channels. We found that 1μM cortisol-BSA had similar effects on the cell surface expression of Kv2.1 as 1μM cortisol in cultured hippocampal neurons (Fig. 6A and B). However, the glucocorticoid receptor inhibitor CORT125281 did not alter the effect of cortisol on Kv2.1 channels (Fig. 6A and B). These data indicate that glucocorticoids regulate cell surface expression of Kv2.1 channels through membrane-associated receptors.

Glucocorticoids regulate Kv2.1 and $Ca_V1.2$ channels through PKA signaling pathway.

Several signaling pathways have been reported for the rapid effects of glucocorticoids through membrane-associated receptors, including PKC, ERK, and especially the cAMP-PKA signaling pathway (37-39). In cultured hippocampal neurons, we observed that 1μM corticosterone rapidly reduced cAMP levels (Fig. 7A) and inhibited PKA activity, as confirmed by a decrease in phosphorylated PKA levels (Fig .7B). Furthermore, the effect of corticosterone on cell surface expression of Kv2.1 channels in hippocampal neurons was mimicked by PKA inhibitor H89 (1 μM) and abrogated by PKA stimulator forskolin (20 μM) (Fig. 7C).

Cortisol inhibited $Ca_V1.2$ currents but had little effect on the cell surface expression of $Ca_V1.2$ in HEK293 cells (Fig. 4), suggesting glucocorticoids regulate $Ca_V1.2$ activity besides promoting the channel endocytosis. Previous studies indicate that PKA can directly phosphorylate $Ca_V1.2$, and this phosphorylation increases $Ca_V1.2$ activity (40). We investigated whether the inhibitory effect of glucocorticoids on $Ca_V1.2$ currents was via the PKA signaling pathway. The PKA inhibitor H89 (1 μM) mimicked the effect of cortisol on $Ca_V1.2$ currents in HEK293 cells (Fig. 7D).

Discussion

This study demonstrates that glucocorticoids regulate Ca^{2+} signals in mammalian hippocampal neurons through the PKA signaling pathway in two ways: by inhibiting PKA to reduce $Ca_V1.2$ channel activities, and by promoting Kv2.1 clusters endocytosis to increase $Ca_V1.2$ channel internalization.

Mammalian brain Kv2.1 channels are typically found in clusters on the somas and proximal dendrites of neurons (41, 42). Increasing evidence suggests that these clustered Kv2.1 channels play important nonconducting roles. Previous studies have shown that Kv2.1 clusters play a structural role in inducing the formation of ER/PM junctions in both cultured hippocampal neurons and transfected HEK293 cells through binding with vesicle-associated membrane protein-associated protein A and B(21, 25) . Clustered Kv2.1 channels at ER/PM junctions recruit $Ca_V1.2$ to form Ca^{2+} microdomains with ryanodine receptor ER Ca^{2+} release channels, which generates local spontaneous Ca^{2+} release at the soma of hippocampal neurons(29). In this study, we found that glucocorticoids rapidly inhibited spontaneous Ca^{2+} sparks. Glucocorticoids not only inhibit $Ca_V1.2$ channel currents, but also disrupt the Ca^{2+} microdomains by targeting clustered Kv2.1 at the soma of cultured hippocampal neurons. The major target of glucocorticoids is clustered Kv2.1 rather than dispersed Kv2.1, as supported by two pieces of evidence. First, glucocorticoids reduced the cell surface expression of Kv2.1 by 30.2%, but had little effect of Kv2.1 currents in HEK293. This can be explained by the fact that clustered Kv2.1 conducts little potassium (43). Second, in the presence of wild-type Kv2.1, glucocorticoids caused a decrease in the surface expression of $Ca_V1.2$ channels in HEK293 cells. However, this effect was not observed in the presence of non-clustering Kv2.1S586A mutant channels.

Neuronal Kv2.1 clusters can be de-clustered by stimuli, including kainite, glutamate, hypoxia, and ischemia(32, 44, 45), while none of them affect the total surface expression of Kv2.1. We found that glucocorticoids affect both the clustering status and surface expression of Kv2.1. This suggests a complex underlying molecular mechanism involved. Previous studies showed that the activation properties of Kv2.1 were altered by channel dephosphorylation, but not by actin depolymerization(43). The actin cytoskeleton is important for forming and maintaining Kv2.1 clusters(46, 47), and TTX has been shown to increase Kv2.1 clustering(48, 49) and stabilize the binding between α-actin and $Ca_V1.2$(50). We found that preincubation with TTX prevented the glucocorticoid induced endocytosis of kv2.1 in HEK293 cells

(supplemental Fig. S1), suggesting that glucocorticoids may act by changing the cell membrane actin skeleton.

The rapid effects of glucocorticoid are commonly mediated by membrane glucocorticoid receptors. We found cortisol-BSA mimicked the effect of cortisol on Kv2.1, while glucocorticoid receptor antagonist, CORT125281, did alter the effect. This suggests that glucocorticoids inhibit Kv2.1 through unknown membrane receptors. Glucocorticoids rapidly suppress PKA in the AtT20 mouse corticotroph tumor cell line(51), but stimulate PKA in HT4 mouse neuroblastoma cells(52), suggesting a cell-specific effect of glucocorticoids on PKA. PKA phosphorylation significantly influences $Ca_V1.2$ activity. Previous studies suggest that AKAP-Anchored PKA is essential for maintaining neuronal $Ca_V1.2$ channel activity and NFAT transcriptional signaling (53). Qian et al. reported that β-adrenergic receptor agonist isoproterenol increases $Ca_V1.2$ activity by stimulating PKA in hippocampal neurons(54). Here, we found that glucocorticoids decreased cAMP level and PKA activity, thereby inhibiting $Ca_V1.2$ currents in hippocampal neurons and HEK293 cells. A recent study showed that glucocorticoids inhibited the levels of cAMP by activation of adhesion G-protein-coupled receptor GPR97(55). Further investigation is needed to determine whether the effect of glucocorticoids reported here is mediated by GPR97.

Glucocorticoids are produced in a diurnal pattern controlled by the hypothalamic -pituitary- adrenal axis. It is now well-known that the glucocorticoid circadian variation is actually composed of an underlying ultradian rhythm. This rhythm occurs less than 60 minutes for rodents(56) and every 60-120 minutes in human(57, 58). Furthermore, the ultradian rhythm of glucocorticoids is not affected by the disruption of circadian inputs to the hypothalamic-pituitary-adrenal activity(59). Recent studies show that the ultradian rhythmicity of glucocorticoids is critical in regulating normal emotional and cognitive responses in humans(4). Wu et al. have revealed that Kv2.1-organized ER/PM junction sites represent around 12% of the somatic surface in mammalian brain neurons(28). At ER/PM junctions, Kv2.1 control somatic Ca^{2+} signals by recruiting $Ca_V1.2$, thereby regulating neuronal excitation–transcription

coupling(30, 60). Since both Kv2.1 and $Ca_V1.2$ are widely expressed in mammalian brain, the rapid effect of glucocorticoids on the Kv2.1-$Ca_V1.2$ unit may play an import functional role in the ultradian rhythm of glucocorticoids.

In conclusion, glucocorticoids rapidly regulate Ca^{2+} signals in hippocampal neurons in two ways. First, they inhibit $Ca_V1.2$ channel activity (regardless of whether it is coupled with Kv2.1 or not) by reducing PKA activity, which results in a reduction of the overall Ca^{2+} signal. Second, they reduce the surface expression of $Ca_V1.2$ channels by promoting the endocytosis of Kv2.1 channels. This disruption of clustered Kv2.1 mediated Ca^{2+} reduces the local spontaneous somatic Ca^{2+} signals that would otherwise regulate excitation-transcription coupling. This study provides further insight into the rapid effect of glucocorticoids in the central nervous system.

Materials and Methods

Cell culture and transfection.

Human embryonic kidney (HEK293) cells were obtained from the cell bank of the Chinese Academy of Science. These cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum and 1% antibiotic antimycotic solution**.** Plasmids for rat GFP-tagged Kv2.1 channels, and mouse $Ca_V1.2$ with β_3 and $\alpha_2\delta_1$ subunits were transiently transfected into HEK293 cells using jetPRIME (Polyplus). For patch clamp recordings, the cells were used after 24 h transfection. Hippocampal neurons from Sprague-Dawley rats of either sex on postnatal day 0 to 1 were used for primary culture. Briefly, hippocampi were dissected and dissociated into single cells with trypsin, and then the cells were plated onto poly-L-lysine–coated glass coverslips inside a six well plate. All experiments were conducted on neurons between 7 and 14 days in vitro (DIV), and were performed on neurons from a minimum of three separate cultures.

All protocols used were approved by the Committee on the Ethics of Animal Experiments of Fudan University, and were in strict accordance with the NIH Guidelines for the Care and Use of Animals.

Molecular biology

Plasmids for rat GFP-tagged Kv2.1 (34) channels, and mouse $Ca_V1.2$(61) were as previously reported. The Kv2.1S586A point mutant was generated using the GFP-Kv2.1 wild-type (WT) plasmid as a template. Site-directed S586A mutagenesis was performed on the Kv2.1 channel using the Quick-Change XL Site-directed Mutagenesis kit (Stratagene, La Jolla, CA, USA). Kv2.1S586A mutation was confirmed by sequencing.

Live cell imaging

Hippocampal neurons and HEK293 cells (transfected with GFP-tagged Kv2.1) were plated onto poly-L-lysine–coated glass bottom dishes. Imaging was performed in HBSS (14025092, Gibco, NY, USA) solution with 20 mM HEPES (H3375, Sigma-Aldrich, Munich, Germany). Hippocampal neurons were first incubated in regular culture medium with 2 μM fluorescent calcium indicator Cal-590 AM (#20510, AAT Bioquest, CA, USA) for 60 minutes at 37 °C. Dye-containing medium was then aspirated, followed by two washes in HBSS which had been warmed to 37 °C. Cells were then incubated in HBSS for an additional 30 minutes at 37 °C prior to imaging. Images were acquired on a Nikon Eclipse Ti TIRF/widefield microscope equipped with an Andor iXon EMCCD camera and a Nikon LUA4 laser launch with 405, 488, 561, and 647 nm lasers, using a 100x/1.49 NA PlanApo TIRF objective and NIS Elements software. Images were analyzed by Fiji (https://fiji.sc). The Fiji plugin xySpark (62) was used for automated spark detection and analysis.

Western blot

The surface proteins of HEK293 and hippocampal cells were biotin-labeled and isolated using a Pierce cell surface protein biotinylation and isolation kit (A44390, Thermo Scientific, IL, USA). The protein samples were resolved using 10% SDS PAGE and transferred to polyvinylidene fluoride membranes. The membranes were immunoblotted with anti-Kv2.1 antibody (1:1000, ab192761, Abcam, Cambridge, UK), anti-$Ca_V1.2$ antibody (1:1000, ab84814, Abcam), anti-Na^+-K^+ ATPase (1:1000,

ab76020, Abcam), anti-beta-tubulin antibody (1:2000, ab179511, Abcam), and anti-PKA antibody (1:1000, #4782, Cell Signaling Technology, MA, USA) and anti-phospho-PKA antibody (1:1000, #5661, Cell Signaling Technology). The blots were developed using enhanced chemiluminescence reagents and imaged using either the ChemiDoc XRS+ imaging system from Bio-Rad (Hercules, CA, USA) or the e-BLOT Touch Imager (e-BLOT Life Sciences, Shanghai, China) with manufacturer´s software. Molecular weight marker and chemiluminescent signal images were automatically overlaid by the manufacturer´s software. Quantitative analysis of detected bands was performed using Fiji (https://fiji.sc/).

Immunofluorescence

Hippocampal neurons on glass coverslips were fixed in 4% paraformaldehyde for 15 minutes at room temperature, washed with cold PBS, and incubated in 0.5% Tween-20 in PBS for 10 minutes. Next, the neurons were blocked with 10% horse serum, 0.3% Triton X-100 in PBS for two hours at room temperature. The primary antibodies used were mouse anti-Kv2.1 (1:500, ab192761, Abcam) and rabbit $Ca_V1.2$ (1:500, ab234438, Abcam), and they were incubated with the cells for 1 day at 4°C in a solution containing 1% horse serum and 0.3% PBST. Cells were washed three times with PBS and incubated overnight at 4°C in a secondary antibody solution containing Cy3-labeled Goat Anti-Mouse/Rabbit IgG(H+L) or Alexa Fluor 488-labeled Goat Anti-Mouse IgG(H+L) (1:500, Beyotime, Shanghai, China), 1% horse serum, and 0.3% PBST. Following another wash with PBS, the cells were imaged using the Nikon A1+ Confocal Microscope System (Tokyo, Japan). The images were subjected to rolling ball background subtraction and subsequently converted into a binary mask by thresholding. The sizes of Kv2.1 clusters were measured using the "analyze particles" feature of Fiji.

cAMP assay

Cultured hippocampal neurons were treated with 1 μM corticosterone for 1, 5 and 10 minutes, respectively. The cells were lysed in 0.1 M HCl. cAMP levels in

hippocampal neurons were measured using a cAMP ELISA Kit (NewEast Bioscience, China) following the manufacturer's instructions.

Electrophysiology

Whole-cell currents in cultured hippocampal neurons and HEK293 cells were recorded using an Axopatch 200B amplifier (Axon Instruments, Union City, CA, USA) and data were collected with PCLAMP 10.7 software (Axon Instruments). The bath solution for potassium current recording contained (in mM): 140 NaCl, 2.5 KCl, 0.001 TTX and 5 4-AP, 10 glucose, 10 HEPES, and 1 $MgCl_2$ (pH adjusted to 7.4 using NaOH). The internal solution contained (in mM): 135 potassium gluconate, 10 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 2 Mg-ATP, and 10 EGTA (pH adjusted to 7.3 using KOH). For calcium current recording, the bath solution contained (in mM): 140 TEACl, 10 $BaCl_2$, 0.001 TTX and 5 4-AP, 10 glucose, 10 HEPES, and 2 $MgCl_2$ (pH adjusted to 7.4 using TEAOH). The internal solution contained (in mM): 125 Cs-methanesulfonate, 1 $MgCl_2$, 10 HEPES, 5 Mg-ATP, 0.3 Tris-GTP and 10 EGTA (pH adjusted to 7.2 using CsOH). The pipettes were made from capillary tubing (BRAND, Wertheim, Germany) and had resistances of 4 to 6 MΩ under these solution conditions. Currents were sampled at 10 kHz and filtered at 2 kHz, and corrected online for leak and residual capacitance transients using a P/4 protocol. All recordings were performed at room temperature.

Cortisol-BSA conjugate (CSB-MC00371b0105) was purchased from CUSABIO (Houston, TX, USA). Cortisol (# B1951), H89 (#B2190), and Forskolin (#B1421) were purchased from APExBIO (Houston, TX, USA). Corticosterone (#HY-B1618) was purchased from MedChemExpress (NJ, USA). All drugs were dissolved in DMSO with a final concentration not exceeding 0.1%.

Statistical analysis

Data analysis was performed using Clampfit 10.7 (Axon Instruments) and GraphPad Prism (v9.4, GraphPad Software Inc, USA). Normality of the data was checked by the Shapiro-Wilk test. The two-tailed paired or unpaired *t*-test was used to compare two

samples, and one-way ANOVA with Bonferroni *post hoc* test was employed for comparing multiple samples. Data are presented as means ± SEM, where 'n' indicates the number of tested cells or independent tests. $P < 0.05$ was considered statistically significant.

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References

1. F. Spiga, J. J. Walker, J. R. Terry, S. L. Lightman, HPA axis-rhythms. *Compr Physiol* **4**, 1273-1298 (2014).
2. X. X. Qian, S. K. Droste, S. L. Lightman, J. M. H. M. Reul, A. C. E. Linthorst, Circadian and Ultradian Rhythms of Free Glucocorticoid Hormone Are Highly Synchronized between the Blood, the Subcutaneous Tissue, and the Brain. *Endocrinology* **153**, 4346-4353 (2012).
3. K. Kalafatakis, G. M. Russell, S. L. Lightman, MECHANISMS IN ENDOCRINOLOGY: Does circadian and ultradian glucocorticoid exposure affect the brain? *Eur J Endocrinol* **180**, R73-R89 (2019).
4. K. Kalafatakis *et al.*, Ultradian rhythmicity of plasma cortisol is necessary for normal emotional and cognitive responses in man. *Proc Natl Acad Sci U S A* **115**, E4091-E4100 (2018).
5. B. A. Simms, G. W. Zamponi, Neuronal voltage-gated calcium channels: structure, function, and dysfunction. *Neuron* **82**, 24-45 (2014).
6. G. W. Zamponi, J. Striessnig, A. Koschak, A. C. Dolphin, The Physiology, Pathology, and Pharmacology of Voltage-Gated Calcium Channels and Their Future Therapeutic Potential. *Pharmacological reviews* **67**, 821-870 (2015).
7. F. Hofmann, V. Flockerzi, S. Kahl, J. W. Wegener, L-type CaV1.2 calcium channels: from in vitro

482 findings to in vivo function. *Physiol Rev* **94**, 303-326 (2014).
483 8. M. J. Sinnegger-Brauns *et al.*, Expression and 1,4-dihydropyridine-binding properties of brain
484 L-type calcium channel isoforms. *Mol Pharmacol* **75**, 407-414 (2009).
485 9. B. Schlick, B. E. Flucher, G. J. Obermair, Voltage-activated calcium channel expression profiles
486 in mouse brain and cultured hippocampal neurons. *Neuroscience* **167**, 786-798 (2010).
487 10. J. W. Hell *et al.*, Identification and differential subcellular localization of the neuronal class C
488 and class D L-type calcium channel alpha 1 subunits. *J Cell Biol* **123**, 949-962 (1993).
489 11. N. C. Clark *et al.*, Neurological phenotype and synaptic function in mice lacking the CaV1.3
490 alpha subunit of neuronal L-type voltage-dependent Ca2+ channels. *Neuroscience* **120**,
491 435-442 (2003).
492 12. S. Moosmang *et al.*, Role of hippocampal Cav1.2 Ca2+ channels in NMDA
493 receptor-independent synaptic plasticity and spatial memory. *J Neurosci* **25**, 9883-9892
494 (2005).
495 13. S. J. Temme, R. Z. Bell, G. L. Fisher, G. G. Murphy, Deletion of the Mouse Homolog of
496 CACNA1C Disrupts Discrete Forms of Hippocampal-Dependent Memory and Neurogenesis
497 within the Dentate Gyrus. *eNeuro* **3** (2016).
498 14. J. Johnston, I. D. Forsythe, C. Kopp-Scheinpflug, Going native: voltage-gated potassium
499 channels controlling neuronal excitability. *J Physiol* **588**, 3187-3200 (2010).
500 15. G. A. Gutman *et al.*, International Union of Pharmacology. LIII. Nomenclature and molecular
501 relationships of voltage-gated potassium channels. *Pharmacological reviews* **57**, 473-508
502 (2005).
503 16. H. Murakoshi, J. S. Trimmer, Identification of the Kv2.1 K+ channel as a major component of
504 the delayed rectifier K+ current in rat hippocampal neurons. *J Neurosci* **19**, 1728-1735 (1999).
505 17. D. P. Mohapatra *et al.*, Regulation of intrinsic excitability in hippocampal neurons by
506 activity-dependent modulation of the KV2.1 potassium channel. *Channels (Austin)* **3**, 46-56
507 (2009).
508 18. P. D. Fox, R. J. Loftus, M. M. Tamkun, Regulation of Kv2.1 K(+) conductance by cell surface
509 channel density. *J Neurosci* **33**, 1259-1270 (2013).
510 19. P. W. Liu, B. P. Bean, Kv2 channel regulation of action potential repolarization and firing
511 patterns in superior cervical ganglion neurons and hippocampal CA1 pyramidal neurons. *J*
512 *Neurosci* **34**, 4991-5002 (2014).
513 20. D. Guan, W. E. Armstrong, R. C. Foehring, Kv2 channels regulate firing rate in pyramidal
514 neurons from rat sensorimotor cortex. *J Physiol* **591**, 4807-4825 (2013).
515 21. P. D. Fox *et al.*, Induction of stable ER-plasma-membrane junctions by Kv2.1 potassium
516 channels. *Journal of cell science* **128**, 2096-2105 (2015).
517 22. H. I. Bishop *et al.*, Kv2 Ion Channels Determine the Expression and Localization of the
518 Associated AMIGO-1 Cell Adhesion Molecule in Adult Brain Neurons. *Front Mol Neurosci* **11**,
519 1 (2018).
520 23. B. Johnson *et al.*, Kv2 potassium channels form endoplasmic reticulum/plasma membrane
521 junctions via interaction with VAPA and VAPB. *Proc Natl Acad Sci U S A* **115**, E7331-E7340
522 (2018).
523 24. M. Kirmiz *et al.*, Remodeling neuronal ER-PM junctions is a conserved nonconducting
524 function of Kv2 plasma membrane ion channels. *Molecular biology of the cell* **29**, 2410-2432
525 (2018).

526 25. M. Kirmiz, N. C. Vierra, S. Palacio, J. S. Trimmer, Identification of VAPA and VAPB as Kv2
527 Channel-Interacting Proteins Defining Endoplasmic Reticulum-Plasma Membrane Junctions in
528 Mammalian Brain Neurons. *J Neurosci* **38**, 7562-7584 (2018).
529 26. E. Okeke, H. Dingsdale, T. Parker, S. Voronina, A. V. Tepikin, Endoplasmic reticulum-plasma
530 membrane junctions: structure, function and dynamics. *J Physiol* **594**, 2837-2847 (2016).
531 27. A. Gallo, C. Vannier, T. Galli, Endoplasmic Reticulum-Plasma Membrane
532 Associations:Structures and Functions. *Annu Rev Cell Dev Biol* **32**, 279-301 (2016).
533 28. Y. Wu *et al.*, Contacts between the endoplasmic reticulum and other membranes in neurons.
534 *Proc Natl Acad Sci U S A* **114**, E4859-E4867 (2017).
535 29. N. C. Vierra, M. Kirmiz, D. van der List, L. F. Santana, J. S. Trimmer, Kv2.1 mediates spatial and
536 functional coupling of L-type calcium channels and ryanodine receptors in mammalian
537 neurons. *Elife* **8** (2019).
538 30. N. C. Vierra, S. C. O'Dwyer, C. Matsumoto, L. F. Santana, J. S. Trimmer, Regulation of neuronal
539 excitation-transcription coupling by Kv2.1-induced clustering of somatic L-type Ca(2+)
540 channels at ER-PM junctions. *Proc Natl Acad Sci U S A* **118** (2021).
541 31. D. E. Antonucci, S. T. Lim, S. Vassanelli, J. S. Trimmer, Dynamic localization and clustering of
542 dendritic KV2.1 voltage-dependent potassium, channels in developing hippocampal neurons.
543 *Neuroscience* **108**, 69-81 (2001).
544 32. H. Misonou, D. P. Mohapatra, M. Menegola, J. S. Trimmer, Calcium- and metabolic
545 state-dependent modulation of the voltage-dependent Kv2.1 channel regulates neuronal
546 excitability in response to ischemia. *J Neurosci* **25**, 11184-11193 (2005).
547 33. D. P. Mohapatra, J. S. Trimmer, The Kv2.1 C terminus can autonomously transfer Kv2.1-like
548 phosphorylation-dependent localization, voltage-dependent gating, and muscarinic
549 modulation to diverse Kv channels. *J Neurosci* **26**, 685-695 (2006).
550 34. Y. Zhang, X. Zhang, C. Liu, C. Hu, Regulation of K(+) Conductance by a Hydrogen Bond in Kv2.1,
551 Kv2.2, and Kv1.2 Channels. *Membranes* **11** (2021).
552 35. S. Berjukow *et al.*, Endogenous calcium channels in human embryonic kidney (HEK293) cells.
553 *Br J Pharmacol* **118**, 748-754 (1996).
554 36. S. T. Lim, D. E. Antonucci, R. H. Scannevin, J. S. Trimmer, A novel targeting signal for proximal
555 clustering of the KV2.1 K+ channel in hippocampal neurons. *Neuron* **25**, 385-397 (2000).
556 37. R. A. Panettieri *et al.*, Non-genomic Effects of Glucocorticoids: An Updated View. *Trends*
557 *Pharmacol Sci* **40**, 38-49 (2019).
558 38. C. Das, M. Thraya, M. M. Vijayan, Nongenomic cortisol signaling in fish. *Gen Comp Endocrinol*
559 **265**, 121-127 (2018).
560 39. F. J. Nunez *et al.*, Glucocorticoids rapidly activate cAMP production via Galphas to initiate
561 non-genomic signaling that contributes to one-third of their canonical genomic effects. *FASEB*
562 *journal : official publication of the Federation of American Societies for Experimental Biology*
563 **34**, 2882-2895 (2020).
564 40. A. Sculptoreanu, E. Rotman, M. Takahashi, T. Scheuer, W. A. Catterall, Voltage-dependent
565 potentiation of the activity of cardiac L-type calcium channel alpha 1 subunits due to
566 phosphorylation by cAMP-dependent protein kinase. *Proc Natl Acad Sci U S A* **90**,
567 10135-10139 (1993).
568 41. J. S. Trimmer, Immunological identification and characterization of a delayed rectifier K+
569 channel polypeptide in rat brain. *Proc Natl Acad Sci U S A* **88**, 10764-10768 (1991).

570 42. T. Kirizs, K. Kerti-Szigeti, A. Lorincz, Z. Nusser, Distinct axo-somato-dendritic distributions of
571 three potassium channels in CA1 hippocampal pyramidal cells. *Eur J Neurosci* **39**, 1771-1783
572 (2014).
573 43. K. M. O'Connell, R. Loftus, M. M. Tamkun, Localization-dependent activity of the Kv2.1
574 delayed-rectifier K+ channel. *Proc Natl Acad Sci U S A* **107**, 12351-12356 (2010).
575 44. H. Misonou *et al.*, Regulation of ion channel localization and phosphorylation by neuronal
576 activity. *Nat Neurosci* **7**, 711-718 (2004).
577 45. H. Misonou, D. P. Mohapatra, J. S. Trimmer, Kv2.1: a voltage-gated k+ channel critical to
578 dynamic control of neuronal excitability. *Neurotoxicology* **26**, 743-752 (2005).
579 46. M. M. Tamkun, M. O'Connell K, A. S. Rolig, A cytoskeletal-based perimeter fence selectively
580 corrals a sub-population of cell surface Kv2.1 channels. *Journal of cell science* **120**, 2413-2423
581 (2007).
582 47. A. V. Weigel, M. M. Tamkun, D. Krapf, Anomalous diffusion of kv2.1 channels observed by
583 single molecule tracking in live cells. *Conference proceedings : ... Annual International*
584 *Conference of the IEEE Engineering in Medicine and Biology Society. IEEE Engineering in*
585 *Medicine and Biology Society. Annual Conference* **2010**, 3005-3008 (2010).
586 48. O. Cerda, J. S. Trimmer, Activity-dependent phosphorylation of neuronal Kv2.1 potassium
587 channels by CDK5. *J Biol Chem* **286**, 28738-28748 (2011).
588 49. S. H. Romer, A. S. Deardorff, R. E. W. Fyffe, A molecular rheostat: Kv2.1 currents maintain or
589 suppress repetitive firing in motoneurons. *J Physiol* **597**, 3769-3786 (2019).
590 50. D. D. Hall *et al.*, Competition between alpha-actinin and Ca(2)(+)-calmodulin controls surface
591 retention of the L-type Ca(2)(+) channel Ca(V)1.2. *Neuron* **78**, 483-497 (2013).
592 51. Y. Iwasaki, Y. Aoki, M. Katahira, Y. Oiso, H. Saito, Non-genomic mechanisms of glucocorticoid
593 inhibition of adrenocorticotropin secretion: possible involvement of GTP-binding protein.
594 *Biochem Biophys Res Commun* **235**, 295-299 (1997).
595 52. J. Z. Han, W. Lin, Y. Z. Chen, Inhibition of ATP-induced calcium influx in HT4 cells by
596 glucocorticoids: involvement of protein kinase A. *Acta Pharmacol Sin* **26**, 199-204 (2005).
597 53. J. G. Murphy *et al.*, AKAP-anchored PKA maintains neuronal L-type calcium channel activity
598 and NFAT transcriptional signaling. *Cell Rep* **7**, 1577-1588 (2014).
599 54. H. Qian *et al.*, Phosphorylation of Ser1928 mediates the enhanced activity of the L-type Ca2+
600 channel Cav1.2 by the beta2-adrenergic receptor in neurons. *Sci Signal* **10** (2017).
601 55. Y. Q. Ping *et al.*, Structures of the glucocorticoid-bound adhesion receptor GPR97-Go complex.
602 *Nature* **589**, 620-626 (2021).
603 56. F. Spiga *et al.*, ACTH-dependent ultradian rhythm of corticosterone secretion. *Endocrinology*
604 **152**, 1448-1457 (2011).
605 57. A. Gavrila *et al.*, Diurnal and ultradian dynamics of serum adiponectin in healthy men:
606 comparison with leptin, circulating soluble leptin receptor, and cortisol patterns. *J Clin*
607 *Endocrinol Metab* **88**, 2838-2843 (2003).
608 58. G. M. Russell *et al.*, Rapid glucocorticoid receptor-mediated inhibition of
609 hypothalamic-pituitary-adrenal ultradian activity in healthy males. *J Neurosci* **30**, 6106-6115
610 (2010).
611 59. E. J. Waite *et al.*, Ultradian corticosterone secretion is maintained in the absence of circadian
612 cues. *The European journal of neuroscience* **36**, 3142-3150 (2012).
613 60. E. L. Yap, M. E. Greenberg, Activity-Regulated Transcription: Bridging the Gap between Neural

Activity and Behavior. *Neuron* **100**, 330-348 (2018).
61. T. Yang, M. He, H. Zhang, P. Q. Barrett, C. Hu, L- and T-type calcium channels control aldosterone production from human adrenals. *The Journal of endocrinology* **244**, 237-247 (2020).
62. E. M. Steele, D. S. Steele, Automated detection and analysis of Ca(2+) sparks in x-y image stacks using a thresholding algorithm implemented within the open-source image analysis platform ImageJ. *Biophys J* **106**, 566-576 (2014).

Figure legends

Figure 1. Effect of glucocorticoids on spontaneous Ca^{2+} sparks in cultured rat hippocampal neurons.

(**A)** Representative TIRF images showing somatic Ca^{2+} sparks in hippocampal neurons (loaded with Cal-590 AM) under the vehicle control (0.01% DMSO) condition and subsequently in the presence of 1 μM corticosterone (Cort) in the same hippocampal neuron. Arrows indicate regions of interest (ROIs) where spontaneous Ca^{2+} signals were detected (scale bar: 10 μm) (Supplemental Video S1). **(B)** Fluorescence intensity traces (upper panels) and kymographs (lower panels) corresponding to the two ROIs indicated in panel A. **(C)** Statistics for the frequency, full width at half maximum (FWHM) and amplitude of all spatially distinct localized Ca^{2+} sparks recorded from hippocampal neurons before and after 1 μM corticosterone treatment using a two-tailed paired *t*-test (n = 11). **p = 0.0065; *p = 0.0461; n.s., not significant (p = 0.4463). Each point corresponds to a single neuron. **(D)** Representative examples of immunofluorescence images showing the Kv2.1 channel clusters in rat hippocampal neurons with treatment of 1 μM corticosterone or an equal volume of DMSO (scale bar: 20 μm). **(E)** Statistics for the percent area of Kv2.1 clusters and the number of Kv2.1 clusters in the somatic region of cultured hippocampal neurons from D using a two-tailed unpaired *t* -test (n = 18). ****p < 0.0001, **p = 0.0018.

Figure 2. Effect of glucocorticoids on Ca^{2+} and K^{+} channel currents, and membrane protein expressions in cultured hippocampal neurons.

(A) Left, representative I_{Ba} traces induced by a depolarization pulse from -90 to 0 mV under the vehicle control (0.01% DMSO) condition and subsequently in the presence of 1μM corticosterone in the same hippocampal neuron. Right, statistical analysis of the effect of corticosterone on I_{Ba} using a two-tailed paired *t*-test (n = 5). ****P = 2.8E-5. **(B)** The time course of the I_{Ba} inhibition by corticosterone. **(C)** Left, representative current traces show the inhibitory effect of 1μM corticosterone on I_K. Right, statistics for the amplitude of I_K from Left using a two-tailed paired *t*-test (n = 14). ****P = 3.2E-4. **(D)** Left, representative I_K traces induced by a depolarization pulse from -80 to +50 mV under the vehicle control condition and subsequently in the presence of 1μM corticosterone in the same hippocampal neuron. Right, plot of I_K activation curves in the DMSO (n = 9 for each data point; blue) and corticosterone-treated (n = 9 for each data point; pink) groups. **(E)** Left, representative western blot of the Kv2.1 channel surface expression after 10-min corticosterone treatment (1μM) in the extracellular solution in cultured hippocampal neurons. Right, statistics from 14 independent experiments using a two-tailed unpaired *t*-test. **** P = 2.2E-9. Na^+-K^+ ATPase as a membrane protein loading control. **(F)** Left, representative western blot of the $Ca_V1.2$ channel surface expression after 10-min corticosterone treatment (1μM) in the extracellular solution in cultured hippocampal neurons. Right, statistics from 10 independent experiments using a two-tailed unpaired *t*-test. ****P = 1.7E-7.

Figure 3. Effect of glucocorticoids on Kv2.1 channels in HEK293 cells.

(A) Left, representative Kv2.1 current traces induced by a depolarization pulse from -80 to +40 mV under the vehicle control (0.01% DMSO) condition and subsequently in the presence of 1 μM cortisol (Cort) in the same HEK293 cell. Right, statistics for the amplitude of Kv2.1 current from Left using a two-tailed paired *t*-test (n = 12). n.s., not significant (P = 0.059). **(B)** Left, representative western blot of the Kv2.1 channel surface expression after 10-min cortisol treatment (1 μM) in the extracellular solution in HEK293 cells. Right, statistics from 14 independent experiments using a two-tailed unpaired *t*-test. ****P = 2.7E-8. Na^+-K^+ ATPase as a membrane protein loading

control. **(C)** Left, representative western blot of the cytosol protein level of Kv2.1 channels after 10-min cortisol treatment (1 μM) in the extracellular solution in HEK293 cells. Right, statistics from 8 independent experiments using a two-tailed unpaired *t*-test. ***P = 0.00062. β-tubulin as an internal control. **(D)** Representative TIRF images showing the effect of 1μM cortisol on Kv2.1 clusters in HEK293 cells. **(E)** Left, representative western blot of the Kv2.1S586A mutant channel surface expression after 10-min cortisol treatment in the extracellular solution in HEK293 cells. Right, statistics from 4 independent experiments using a two-tailed unpaired *t*-test. P = 0.5745.

Figure 4. Effect of glucocorticoids on $Ca_V1.2$ channels in HEK293 cells.

(A) Left, representative I_{Ba} traces induced by a depolarization pulse from -90 to 0 mV under the control (0.01% DMSO) condition and subsequently in the presence of 1 μM cortisol in the same HEK293 cell. Right, statistics for the amplitude of Kv2.1 current from Left using a two-tailed paired *t*-test (n = 7). ***P = 8.2E-6. **(B)** I-V curve of $Ca_V1.2$ currents in the absence or presence of 1 μM cortisol in HEK293 cell. **(C)** 1 μM cortisol did not alter the steady-state activation of $Ca_V1.2$ currents. **(D)** Left, representative western blot of the $Ca_V1.2$ channel surface expression after 10-min cortisol treatment (1μM) in the extracellular solution in HEK293 cells. Right, statistics from 4 independent experiments using a two-tailed unpaired *t*-test. n.s., not significant (P = 0.1143).

Figure 5. Effect of glucocorticoids on $Ca_V1.2$ channels in HEK293 cells expressing Kv2.1.

(A) Left, representative western blot of the $Ca_V1.2$ channel surface expression after 10-min cortisol treatment (1μM) in the extracellular solution in HEK293 cells expressing both $Ca_V1.2$ and Kv2.1 channels. Right, statistics from 5 independent experiments using a two-tailed unpaired *t*-test. ***P = 0.0004, **P = 0.00197. **(B)** Left, representative I_{Ba} traces induced by a depolarization pulse from -90 to 0 mV under the control condition and subsequently in the presence of 1μM cortisol in the

same HEK293 cell. Right, statistics for the amplitude of Kv2.1 current from Left using a two-tailed paired *t*-test (n = 5). ***P = 3.4E-5. **(C)** Representative western blots (left) and statistical analysis (right) show the effect of cortisol on the $Ca_V1.2$ channel surface expression when $Ca_V1.2$ and Kv2.1S586A channels are co-expressed in HEK293 cells. Two-tailed unpaired *t*-test, n = 6, P = 0.4339.

Figure 6. Glucocorticoids modulate the cell surface expression of Kv2.1 channels through membrane-associated receptors.

(A) Representative western blot images show the effects of cortisol-BSA and cortisol with/without the glucocorticoid receptor inhibitor CORT125281 on Kv2.1 channel surface expression in cultured hippocampal neurons. (**B)** Statistical analysis of the effects of various treatments on the cell surface expression of Kv2.1 using a one-way ANOVA with Bonferroni *post hoc* test (n = 5). **P < 0.01. n.s., not significant.

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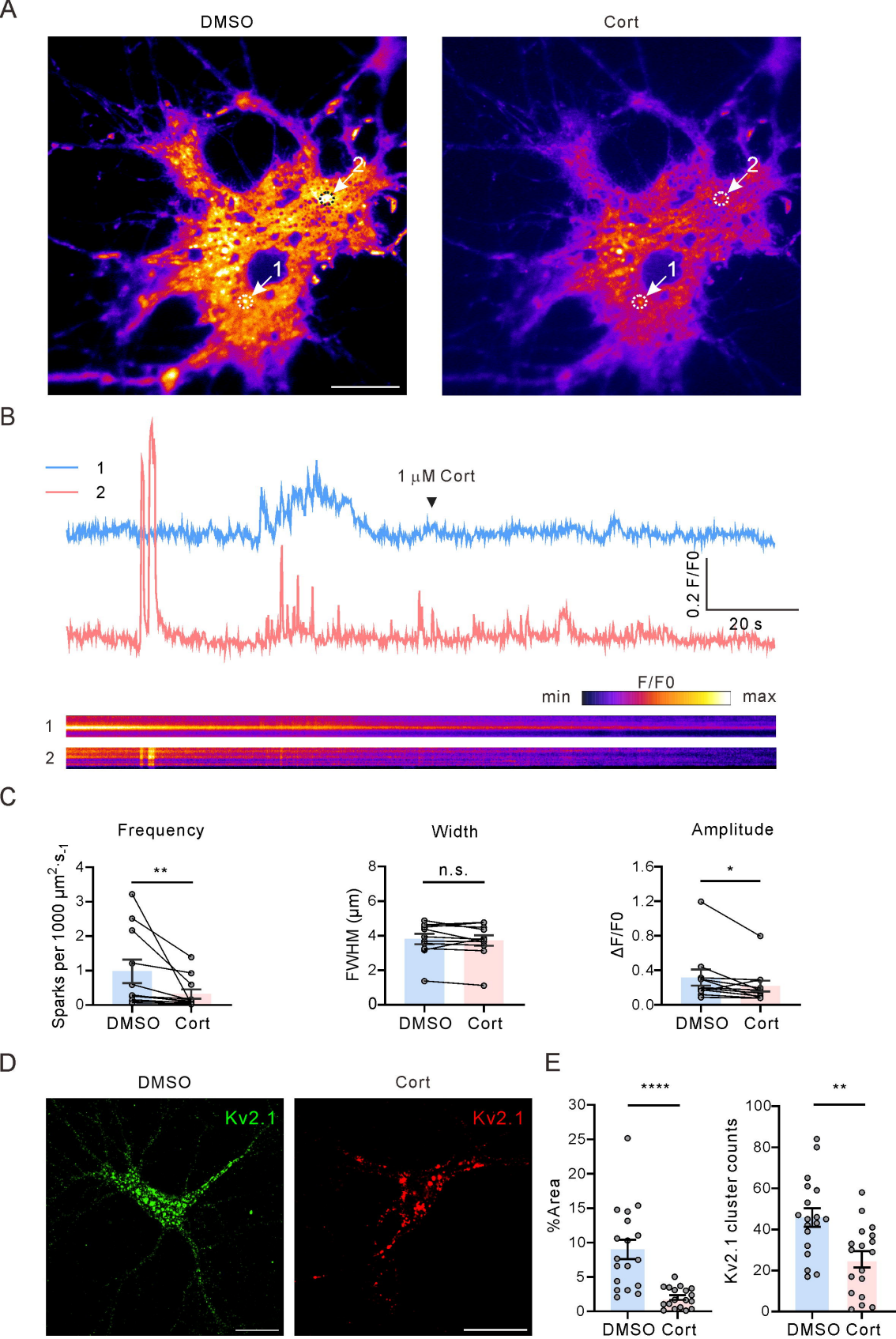
Figure 7. Glucocorticoids reduce the cell surface expression of Kv2.1 and $Ca_V1.2$ channels through PKA signaling pathway

(A) Extracellular application of 1 μM corticosterone rapidly reduced cAMP levels in cultured hippocampal neurons (n = 3, ***P < 0.001, one-way ANOVA with Bonferroni *post hoc* test). **(B)** Left, representative Western blot of the PKA phosphorylation level after 10-min corticosterone treatment in cultured hippocampal neurons. Right, statistics from 7 independent experiments using a two-tailed unpaired *t*-test. *P = 0.0101. **(C)** Representative western blots (left) and statistical analysis (right) showing the effect of PKA inhibitor H89 and agonist Forskolin (Fsk) on the Kv2.1 channel surface expression in cultured hippocampal neurons. n = 6, ****P < 0.0001, **p = 0.0034, n.s., not significant (p = 0.5948), one-way ANOVA with Bonferroni *post hoc* test. **(D)** Left, representative $Ca_V1.2$ current traces induced by a depolarization pulse from -90 to 0 mV under the control condition and subsequently in the presence of 1μM H89 in the same HEK293 cell. Right, statistics for the amplitude of $Ca_V1.2$ current from Left using a two-tailed paired *t*-test (n = 10). ****P < 0.0001.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DMSO
Cort
1
2
B
1 μM Cort
0.2 F/F0
20 s
F/F0
min
max
C
Frequency
Sparks per 1000 μm2·s-1
**
DMSO
Cort
Width
FWHM (μm)
n.s.
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ΔF/F0
*
D
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%Area
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Kv2.1 cluster counts
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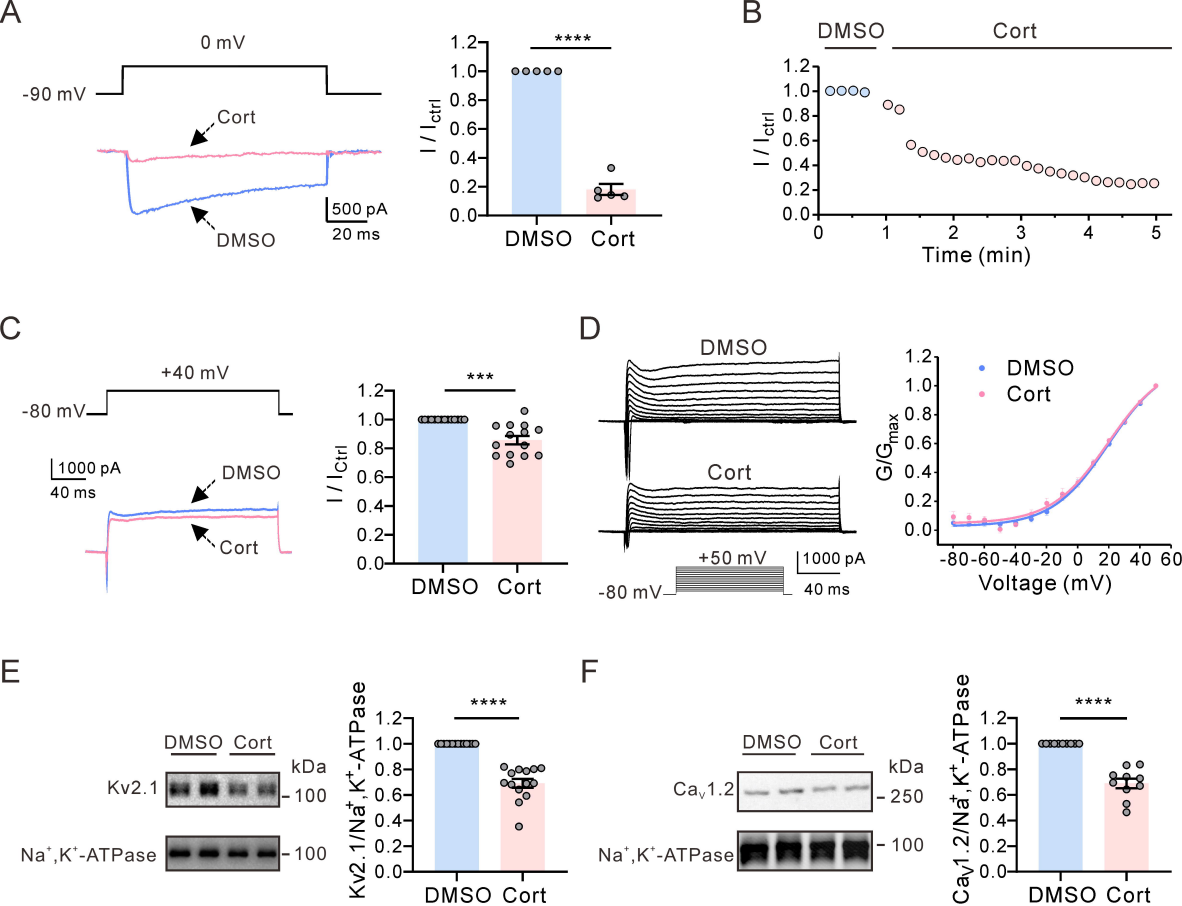
Figure 1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-90 mV
Cort
DMSO
500 pA
20 ms
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DMSO
Cort
B
DMSO
Cort
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Time (min)
C
+40 mV
-80 mV
1000 pA
40 ms
DMSO
Cort
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I / ICtrl
DMSO
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D
DMSO
Cort
+50 mV
-80 mV
1000 pA
40 ms
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G/Gmax
Voltage (mV)
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Kv2.1
100
Na+,K+-ATPase
100
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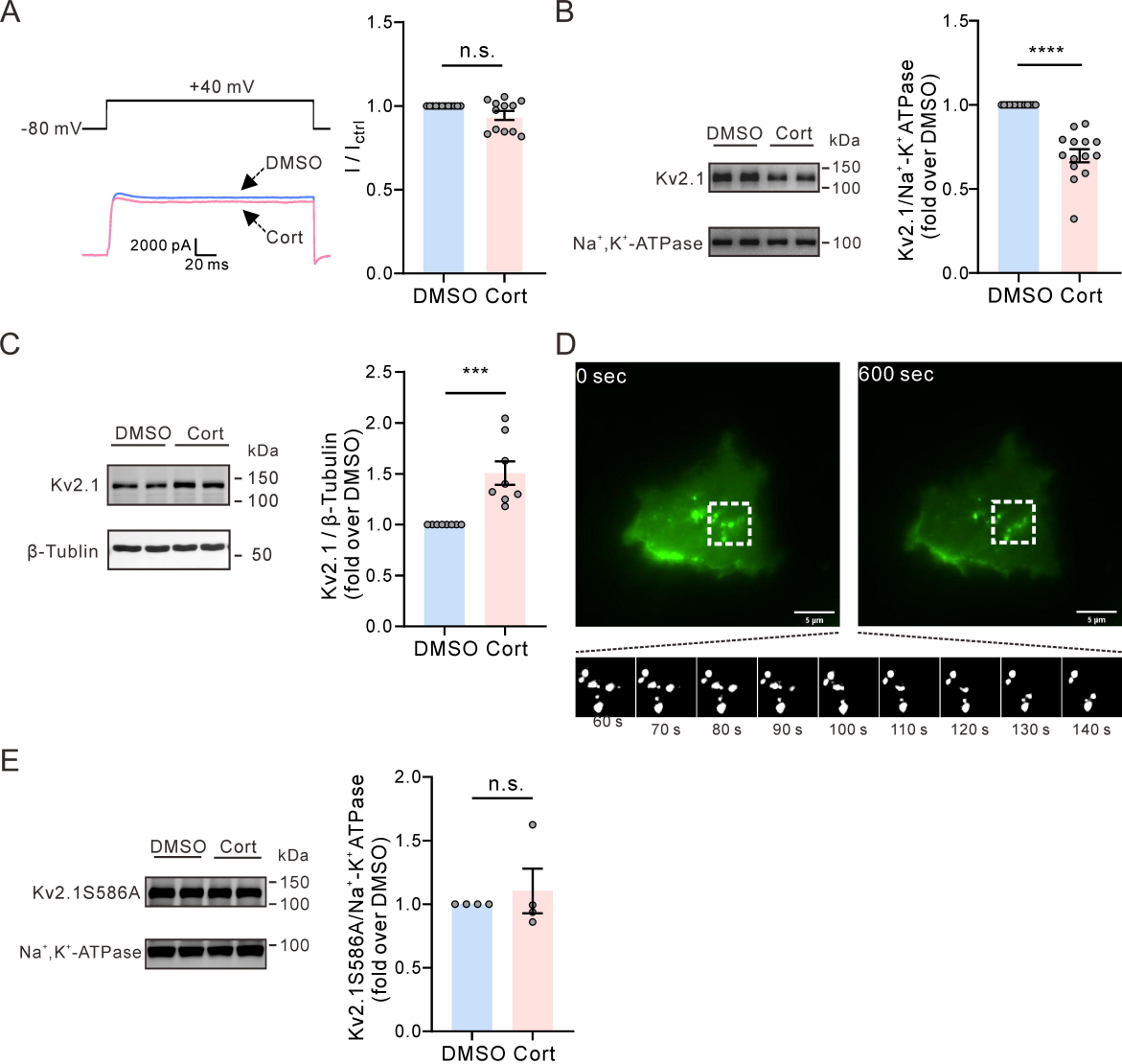
Kv2.1/Na+,K+-ATPase
DMSO
Cort
F
DMSO
Cort
kDa
CaV1.2
250
Na+,K+-ATPase
100
****
CaV1.2/Na+,K+-ATPase
DMSO
Cort

Figure 2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+40 mV
-80 mV
DMSO
Cort
2000 pA
20 ms
n.s.
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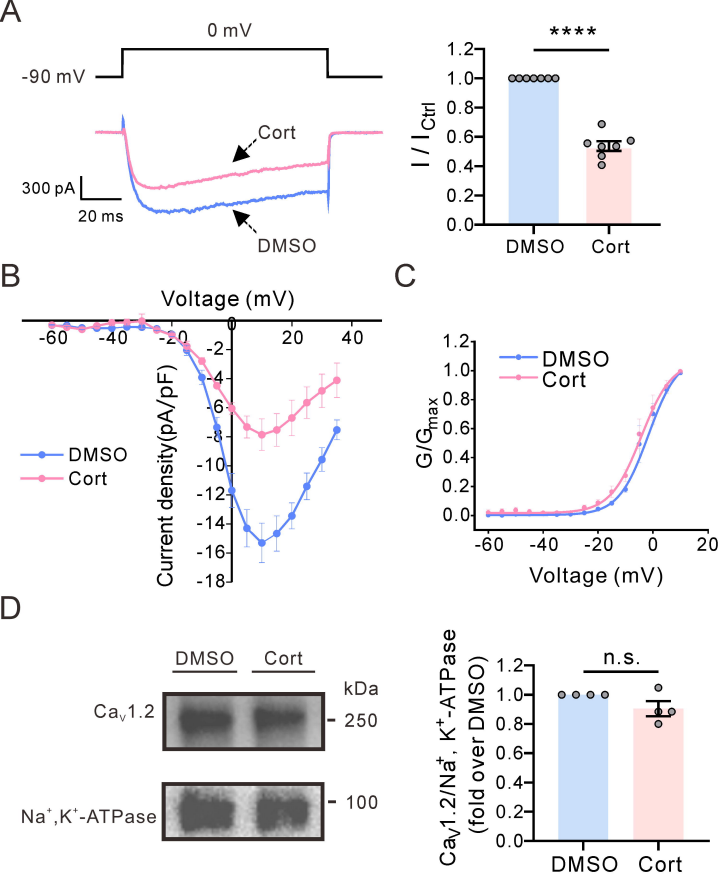
1.5
1.0
0.5
0.0
DMSO Cort
B
DMSO Cort
kDa
Kv2.1
150
100
Na+,K+-ATPase
100
****
Kv2.1/Na+-K+ATPase
(fold over DMSO)
1.5
1.0
0.5
0.0
DMSO Cort
C
DMSO Cort
kDa
Kv2.1
150
100
β-Tublin
50
***
Kv2.1 / β-Tubulin
(fold over DMSO)
2.5
2.0
1.5
1.0
0.5
0.0
DMSO Cort
D
0 sec
600 sec
5 μm
5 μm
60 s
70 s
80 s
90 s
100 s
110 s
120 s
130 s
140 s
E
DMSO Cort
kDa
Kv2.1S586A
150
100
Na+,K+-ATPase
100
n.s.
Kv2.1S586A/Na+-K+ATPase
(fold over DMSO)
2.0
1.5
1.0
0.5
0.0
DMSO Cort

Figure 3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-90 mV
Cort
300 pA
20 ms
DMSO
****
I / I_Ctrl
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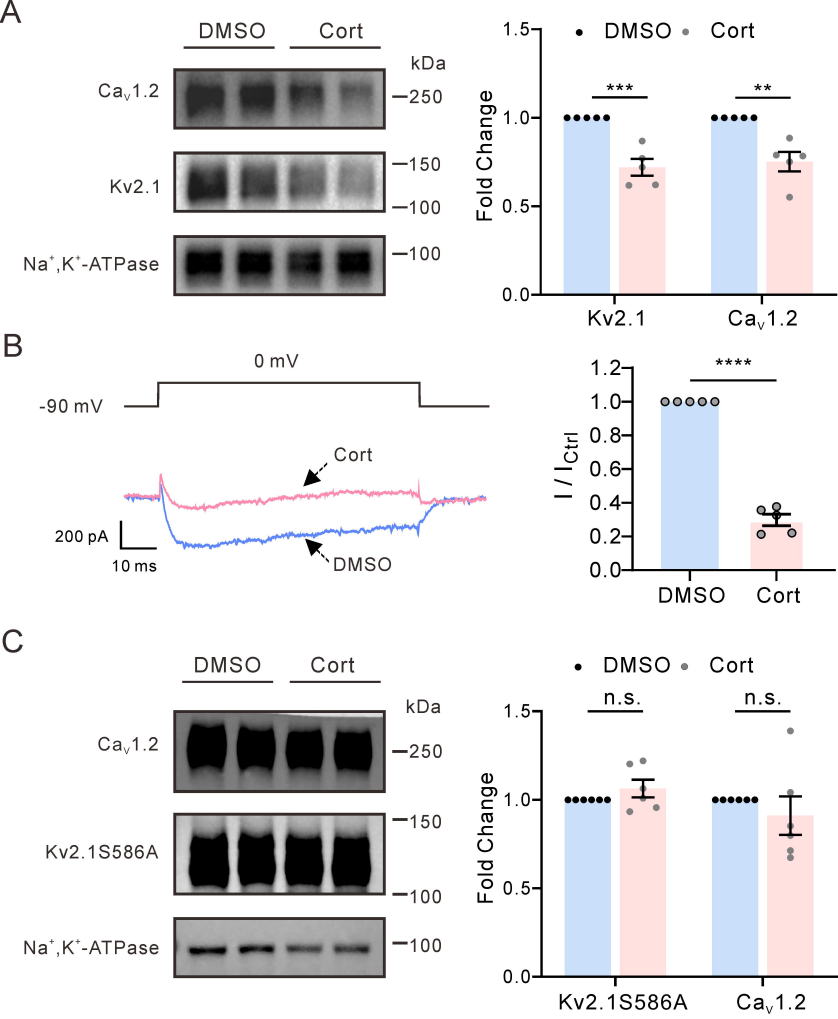
1.0
0.8
0.6
0.4
0.2
0.0
DMSO
Cort
B
Voltage (mV)
-60
-40
-20
0
20
40
-2
-4
-6
-8
-10
-12
-14
-16
-18
Current density(pA/pF)
DMSO
Cort
C
G/G_max
DMSO
Cort
Voltage (mV)
D
DMSO
Cort
kDa
Ca_V1.2
250
Na+,K+-ATPase
100
n.s.
Ca_V1.2/Na+, K+-ATPase
(fold over DMSO)
DMSO
Cort

Figure 4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DMSO
Cort
kDa
Cav1.2
250
Kv2.1
150
100
Na+,K+-ATPase
100
DMSO
Cort
Fold Change
1.5
1.0
0.5
0.0
***
**
Kv2.1
Cav1.2
B
0 mV
-90 mV
Cort
200 pA
10 ms
DMSO
I / ICtrl
1.2
1.0
0.8
0.6
0.4
0.2
0.0
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DMSO
Cort
C
DMSO
Cort
kDa
Cav1.2
250
Kv2.1S586A
150
100
Na+,K+-ATPase
100
DMSO
Cort
n.s.
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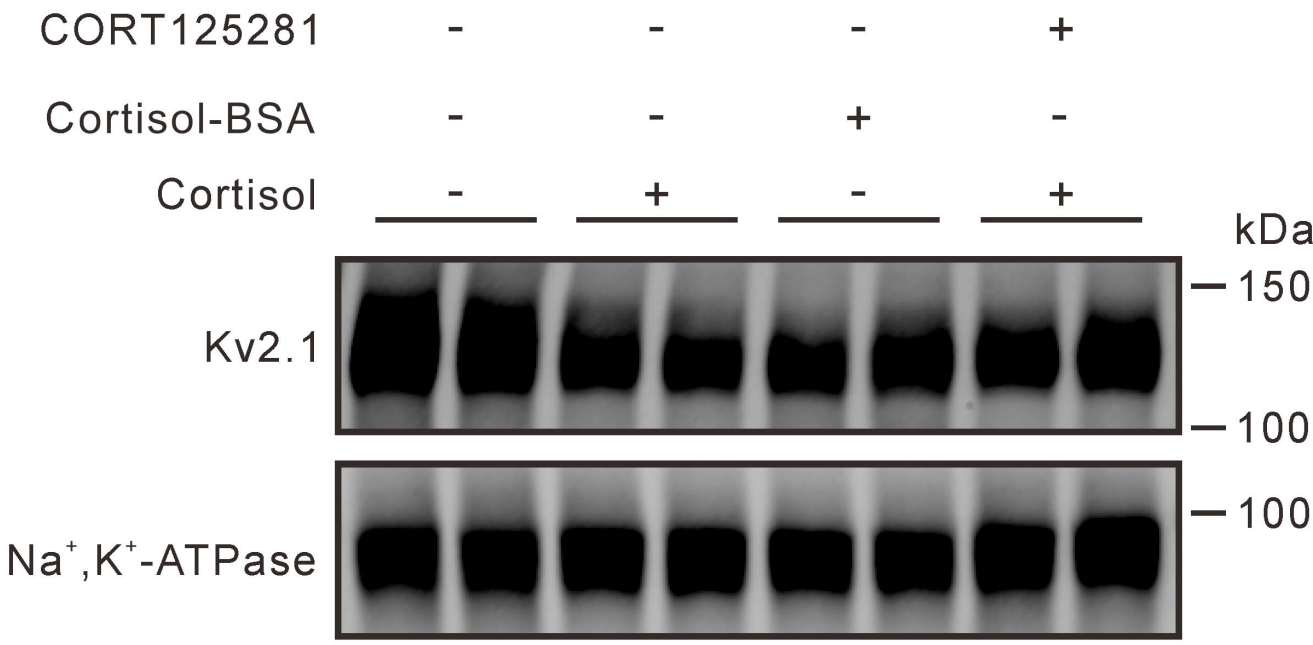
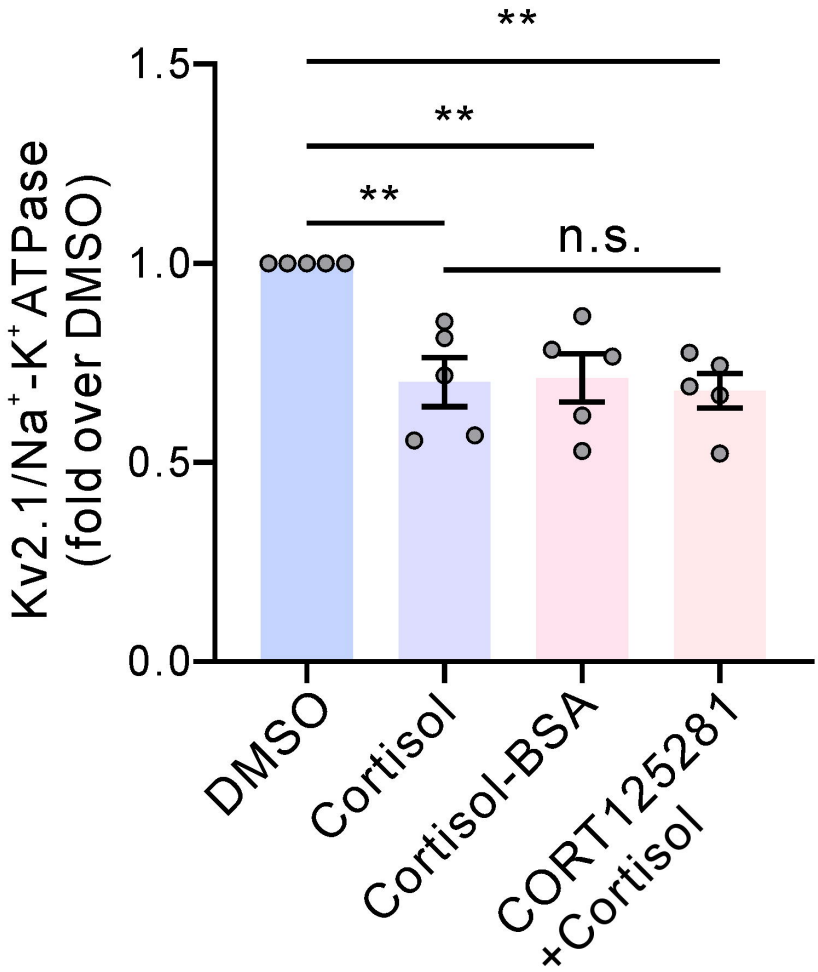
Fold Change
1.5
1.0
0.5
0.0
Kv2.1S586A
Cav1.2

Figure 5

A


CORT125281 - - - +
Cortisol-BSA - - + -
Cortisol - + - +
kDa
Kv2.1
150
100
Na+,K+-ATPase
100

B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(fold over DMSO)
1.5
1.0
0.5
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**
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CORT125281
+Cortisol

Figure 6

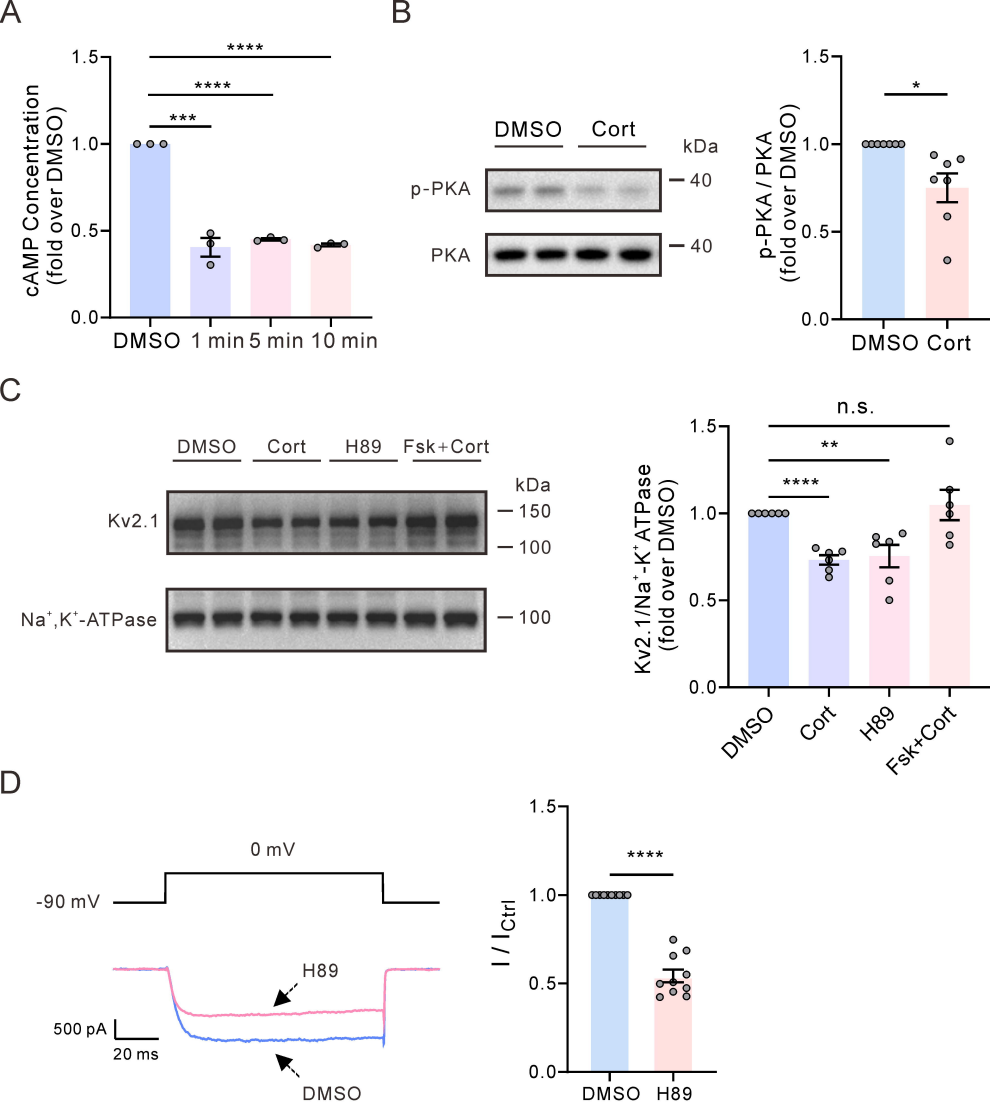
A
cAMP Concentration (fold over DMSO)
1.5
1.0
0.5
0.0
****
****
***
DMSO
1 min
5 min
10 min
B
DMSO
Cort
kDa
p-PKA
PKA
40
40
p-PKA / PKA (fold over DMSO)
*
DMSO
Cort
C
DMSO
Cort
H89
Fsk+Cort
kDa
Kv2.1
150
100
Na+,K+-ATPase
100
Kv2.1/Na+-K+ ATPase (fold over DMSO)
n.s.
**
****
DMSO
Cort
H89
Fsk+Cort
D
0 mV
-90 mV
H89
500 pA
20 ms
DMSO
I / ICtrl
****
DMSO
H89

Figure 7