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Supplementary Information for

Chronic and Acute Stress Monitoring by Electrophysiological Signal from Adrenal Gland

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Materials and Methods

Device Fabrication.

A 5-μm-thick substrate was prepared by spin coating with poly(pyromellitic dianhydride-co-4,4‘-oxydianiline) solution (Sigma-Aldrich) on pre-formed sacrificial layer, a 200 nm thick spin coated PMMA (Microchem) on a glass substrate. 20 nm thick chromium as an adhesive layer, and 50 nm thick gold as a conductive layer were sputtered on polymer-coated glass. The metal layers were patterned using AZ GXR 601 photoresistor (Merck, Germany), followed by wet chemical etching. After that, SU-8 2 photoresist (Microchem) encapsulated Au electrode, except for four contact pads (10 μm × 10 μm) and four I/O pads (250 μm × 8 mm). Contact and I/O pads were to be utilized to measure EP signals from target cells, and to be connected with ACF cable and PCB board, respectively. After patterning of aluminum etching mask with AZ GXR 601 on the device, devices could be isolated and defined by polymer etching down to the bottom glass surface, using oxygen-based reactive-ion-etching (RIE). The exposed PMMA layer under pre-defined devices could be removed by acetone. The 7-μm-thick overall device was then obtained. In parallel, photoresist SU-8 100 (Microchem) on a glass substrate was used for the fabrication of a ~250-μm-thick shuttle. As the design of the probe and the shuttle had the same geometric structure, they were able to be aligned after being stacked together using a mask aligner. SU-8 2 material was used for stacking adhesive between electrodes and shuttle at only anchor for easy release. After that, the overall probe was connected to custom-made PCB board (DITECH, Korea) with conventional FFC (Heat Seal ACF cable, Elform Inc., USA).

Surgical Procedure for the Devices Implantation.

Male rats (Orient bio, Korea) of (7 or 8) weeks old were purchased, and adapted in the conventional level animal facility for about one week. Urethane (Sigma-Aldrich) solution of 1,000 mg/kg was used for animal anesthesia, since we must stabilize rat for a long recording (more than 5 hours). After opening of abdominal cavity by incising the skin of the back, the probe was implanted by penetrating the whole adrenal gland. The implanted probe was drawn back gently, in order to confirm that the probe was fixed with the anchor. After penetration, we held the arrowhead part with forceps, mechanically lifted up the I/O pad part of the thin sensor layer, and broke the shaft part in the shuttle for easy removal. The broken shaft and I/O pad parts were removed by retreating, so that only the sensor layer remained in the tissue. Thereafter, the connector was sutured under the skin with biodegradable Coated Vicryl® suture (Ethicon), leaving only the pins of the connector on the skin.

Measurement of EP Signal.

Rats with probe implanted were anesthetized with urethane. We placed the anesthetized rats on a bed, and left them for about half an hour to eliminate handling stress. Copper tape as a ground electrode was used between the bed and thorax of the rat, in order to collect physical noise, such as breathing and heartbeating. To remove external noise, copper-grid cage was also used. Smartlink™ (NeuroNexus) headstage of Smartbox™ (NeuroNexus), a commercial signal acquisition system, was linked to pins of the connector. The reference electrode of the headstage was lined to metal needle, and poked in the rat abdominal skin, and the copper tape placed on the bed under the animal. The ground electrode was lined to both steel table under the metal grid cage, and the grounding conductor of the receptacle. We changed reference and ground cable into different locations, until external noise was minimized. After eliminating noise, we collected signals from the adrenal gland with sampling rate of 20,000 S/s, after applying notch filter at 60 Hz. The signal was recorded from (30 to 120) minutes, depending on the purpose of the measurement. All EP signal data were achieved by multiple animals and trials of experiment for

injection of ACTH (11 rats), PBS (2 rats), DMSO (5 rats), DMSO+KZ (7 rats), PBS+CHX (4 rats), and fored swimming test (4 rats).

ACTH Injection.

Rats with probe implanted were allowed to recover for 1 week after surgery, and then anesthetized again with urethane solution. The anesthetized animal was kept in cage for 30 minutes, in order to remove the previous effect of stress induced by handling. The probe was then linked to the conventional signal acquisition system, Smartbox™ (Neuronexus), and the signal from the adrenal gland was measured within about 60 minutes. ACTH solution (basic dose: 60 ng/mL) was then injected intravenously. The ACTH-effected signal was recorded for about (60 to 90) minutes, and analyzed by computation program Matlab (Mathworks).

Measurement of Cortisol and Glucose Level in a Blood Sample.

Blood test was done in parallel with signal collection. The animal with the implanted probe was anesthetized with urethane solution. Pins of the connector were linked with commercial headstage. Blood sample for measuring the cortisol level was collected every 30 minutes (two samples before ACTH injection, and three samples after ACTH injection) by venipuncture. The collected blood sample of 0.3 mL was moved into PCR tube, and immediately stored at -70 °C freezer. After collection, the blood sample was moved to an *in vitro* cortisol sensor, and its resistance was measured. Blood cortisol level was calculated by comparing the resistance changes of the cortisol sensor that was previously fabricated. Otherwise, a drop of blood was collected from the tail vein every 15 minutes. The collected blood was dropped on the slit of a conventional glucose level tester, Accu-Chek® (Roche). Blood glucose level was measured three times before ACTH injection, and 4 or more times after ACTH injection.

Cortisol Inhibition.

Rats with inserted probes were stabilized for 1 week after surgery, and then anesthetized again, using urethane. Protein synthesis antagonist cycloheximide (Sigma-Aldrich) 50 μg and ketoconazole (Sigma-Aldrich) 20 mg were intravenously introduced, and the connector was connected to the Smartbox device 6 hours later. In ACTH injection test, the reference signal of adrenal gland was collected for about 60 minutes, and ACTH saline solution (60 ng/mL) was then intravenously injected. The signal was then collected for (60 to 90) minutes.

Forced Swim Test.

Rats with inserted probes were stabilized for 2 weeks after surgery, and then anesthetized again using urethane. The connector was connected to the Smartbox™ device, and the signal from the adrenal gland was measured in about 60 minutes. After the anesthesia was removed, rats were placed in a vessel (diameter 30 cm) containing water with higher height than its own length, so that rats had to swim continuously for 5 minutes. After 5 minutes of forced swim test, rats were rescued from the water vessel, and then anesthetized with urethane. The connector was then connected to the Smartbox™ device to collect signal from the adrenal gland for 60 minutes.

Test for Freely-Moving Animal model.

Rats with the adrenal probe were anesthetized with 10 mg ketamine (Yuhan, Korea), then connected to the SmartBox™ device. The reference signal was collected before the rat woke from the anesthesia. After the animal awoke, we continued the recording, while paying attention so as not to disconnect the connector. The recording stopped within 10 minutes, since the fully awoken animal started to scratch the connector and headstage, and caused bleeding and damage.

Histology Analysis.

Rats with inserted probes were allowed to recover for (1 and 2) week after surgery, and then anesthetized using urethane. The abdominal cavity was incised to open, and the adrenal gland in which the probe was implanted was excised and dispatched. The connected FFC and connecter

were also removed from the body. The excised adrenal glands were immersed in 4 % paraformaldehyde solution (Biosesang, Korea), and kept in 4 °C refrigerator for 24 hours. The density of the tissue was adjusted by soaking the adrenal gland in 30 % sucrose solution (Sigma-Aldrich) for 3 days. The solution was replaced daily. The fixed adrenal gland was sliced into 40 μm in thickness with CM1950 cryocut microtome (Leica, Germany), placed on slide glass, and subjected to ordinary H&E staining. The stained tissue sample was observed using a DM-2700M optical microscope (Leica, Germany).

Open-field Behavior Test.

Two groups of rats were prepared. One group was implanted with the adrenal probe as usual. For the other group, only the abdomen was opened from the dorsal direction. After any further treatment, it was then sutured again. After 4 weeks of recovery, both groups of rats were placed in a dark room for 30 minutes for adaptation. After adaption, each rat was exposed to an open-field of (60 cm × 60 cm) black plastic cage in dark chamber. The mobility of rat was recorded with behavior analyzing device EthoVision XT 10 (Noldus, Netherlands).

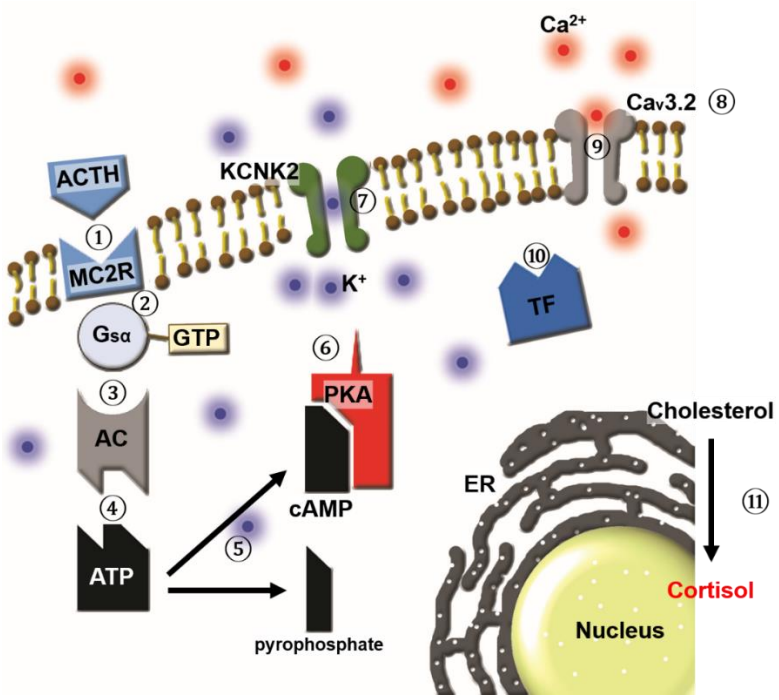

Fig. S1. The molecular biological mechanism of the ACTH-response in AZF cell (3-8). ACTH in bloodstream binds to melanocortin receptor 2 (MC2R) on the surface of adrenal zona fasciculta cell (AZF cell). MC2R-ACTH performs as a guanine nucleotide exchange factor (GEF). It detaches guanosine diphosphate (GDP) from G protein that is linked to MC2R, so that guanosine triphosphate (GTP) can be attached. When GTP is linked, the alpha unit of the G protein is activated, separating from the beta and gamma unit of the G protein. Activated G protein-alpha unit attaches to adenylyl cyclase (AC) to be activated. Activated AC acts as a catalyst that separates adenosine triphosphate (ATP) into pyrophosphate and 3', 5'–cyclic adenosine monophosphate (cAMP). Protein kinase A (PKA) is an enzyme whose activation is strongly influenced by the concentration of cAMP. Therefore, more cAMP in cell activates more PKA to bind to potassium channel (KCNK2 potassium channel), and open the channel. Then potassium ion outflux occurs, because of ion gradient of the cell membrane. This action potential spreads through the cell membrane, and opens $Ca_v3.2$ voltage-gated calcium channel. Calcium ion influx then occurs. Calcium ion in the cell is linked to transcription factor (TF), and activates it. The activated TF moves to endoplasmic reticulum, and transfers cholesterol to cortisol.

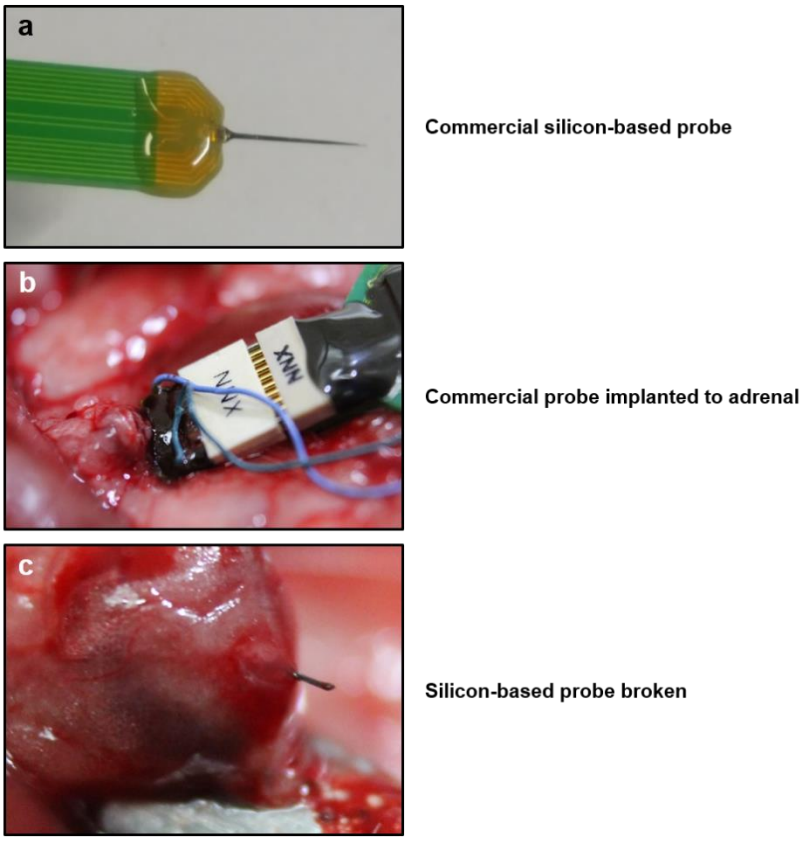

Fig. S2. The implantation of conventional silicon-based probe. Conventional MEMS-based probe is now widely used in neuroscience and brain electrophysiology. However, its application to the adrenal gland is difficult. Since silicon-based probe is brittle and stiff, the probe could not endure dynamic condition in the abdominal cavity. We purchased commercialized silicon probe and implanted it to the brain. The probe easily penetrated dura mater and pia mater of the brain and fixed tightly on the skull. The probe could also be easily implanted on the adrenal gland. However, as soon as the forceps that had fixed the adrenal gland were removed, the probe broke due to natural movement of the tissue.

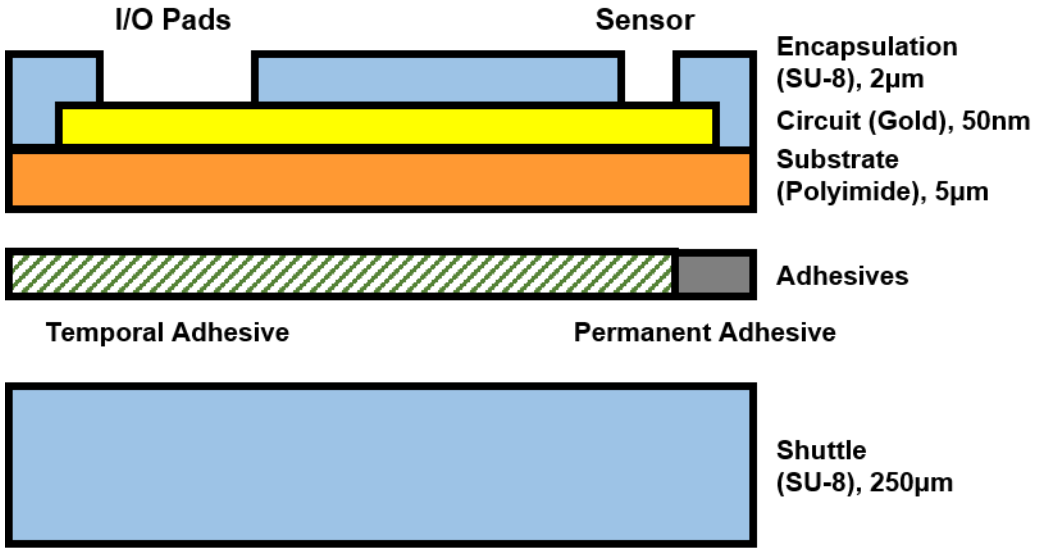

Fig. S3. Probe design and materials for data acquisition. I/O pads contain four wide and large pads. They represent each electrode channel, respectively. One end of the FFC is aligned and attached on the pads, while the other end of the FFC is linked to the custom-made PCB board connector designed to be suitable for the commercialized data analyzing device. The bottom shuttle is made of 250 μm thickness of epoxy (SU-8 50), to provide enough stiffness to be accurately penetrated into the elastic nature of the adrenal gland. Since the shuttle layer was relatively brittle, we fractured the shuttle, and removed it after implantation for biocompatibility in the chronic remaining. Both layers were attached with UV curable adhesive only at the anchor part, while the shaft and I/O pads part were temporarily attached. When the PCB connector was linked to the commercialized headstage device, the signal collected from each electrode was transmitted to the signal-collecting device.

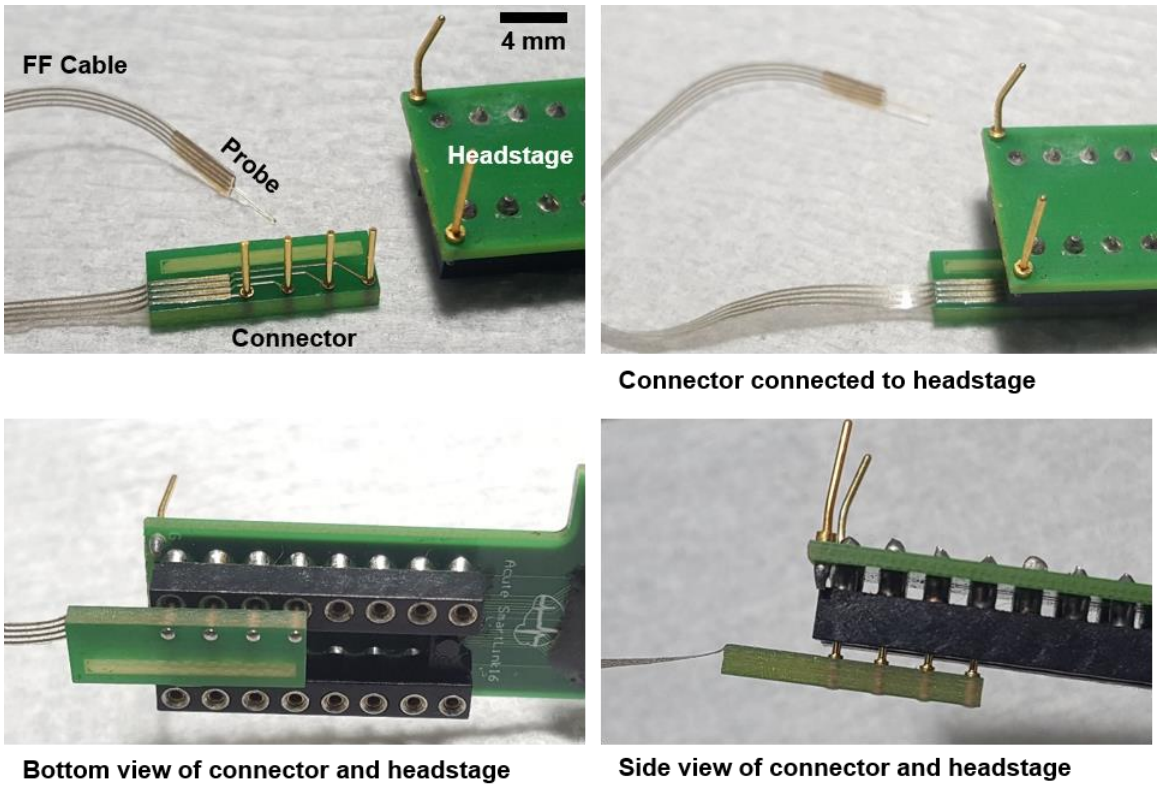

Fig. S4. Images of the PCB connector system. This images show the connector lined to the probe and the commercialized headstage for the data collection device (top left). The connector is linked to the headstage for data transmission (top right). In the case of actual implantation, only the gold-coated pins are exposed to the skin surface, while the whole device including the probe with FFC is immerged in the skin. Bottom view (bottom left) and side view (bottom right) of the probe connected to the headstage.

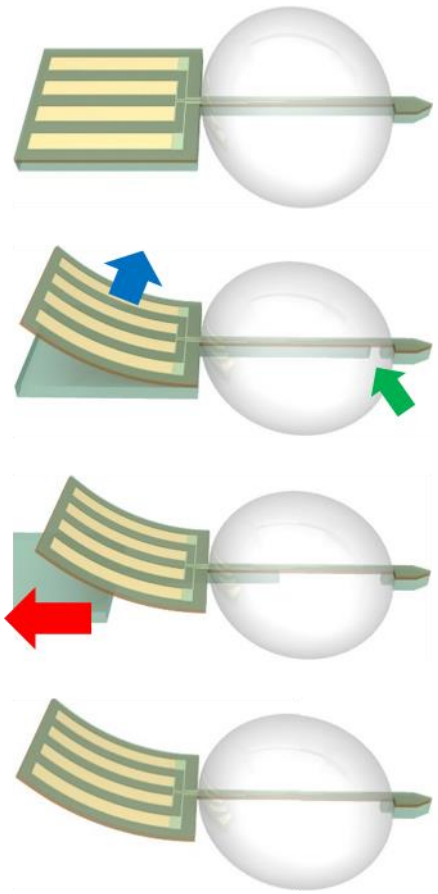

Fig. S5. Penetrated sensor procedure into the adrenal gland. Flexible sensor attached with rigid brittle shuttle was implanted through the adrenal gland (white transparent sphere). After the penetration, the temporal adhesive was removed (blue arrow). Then we mechanically broke the neck, and separated the rigid materials from the implanted sensors (green arrow). The broken shuttle was removed by retreating with forceps (red arrow), leaving the arrowhead anchor part that was holding the adrenal capsule.

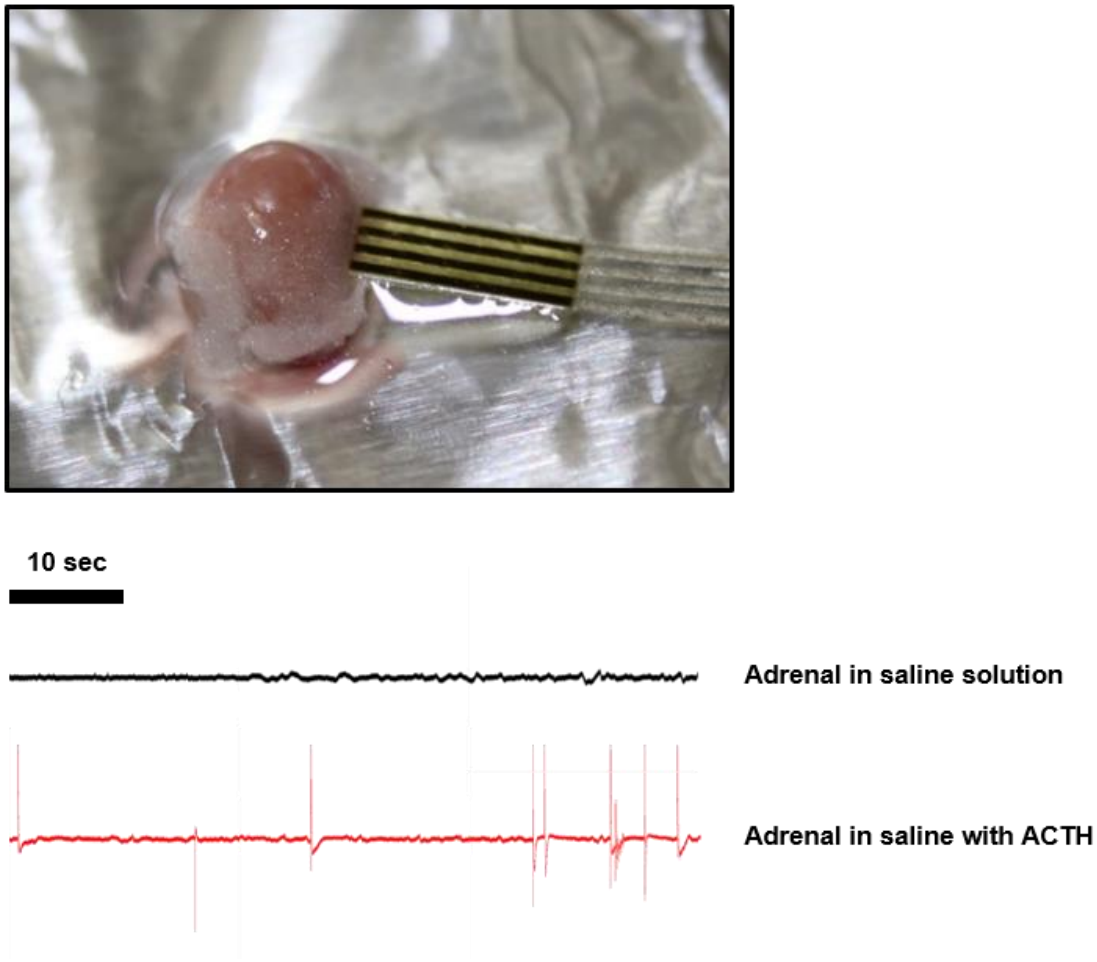

Fig. S6. *In vitro* experiment of adrenal probe. The image of the probe implanted into the excised adrenal gland (top) is shown. The excised adrenal gland was immersed in the petri dish filled with saline solution. *In vitro* signal was collected with the probe, before (black) and after (red) ACTH was released in the saline solution (bottom). Clear differences in signal were observed after the ACTH injection.

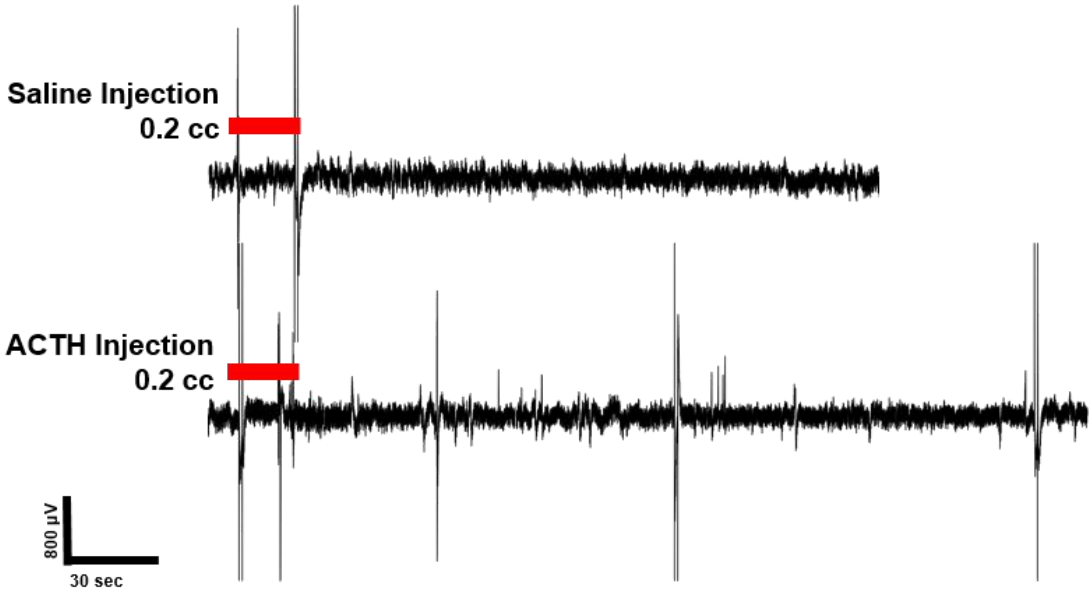

Fig. S7 Continuous recording of the adrenocortical signal after 0.2 cc ACTH injection. When we injected 0.2 cc saline solution (top), there was no signal change in the adrenal gland. When we injected the same amount of ACTH solution, the signal was evoked in the adrenal gland within a few seconds.

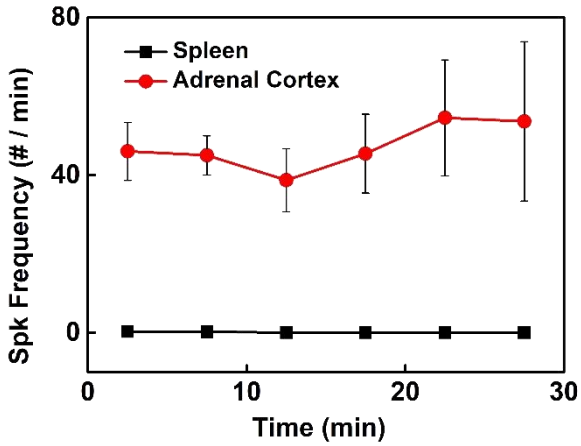

Fig. S8. Time-course spike frequency of adrenal gland and spleen after ACTH administration. Signal acquired simultaneously on both adrenal gland and spleen after ACTH injection. After ACTH injection, action potential-like spikes were only recorded on the adrenal gland (red line), while the spleen was silenced (black line).

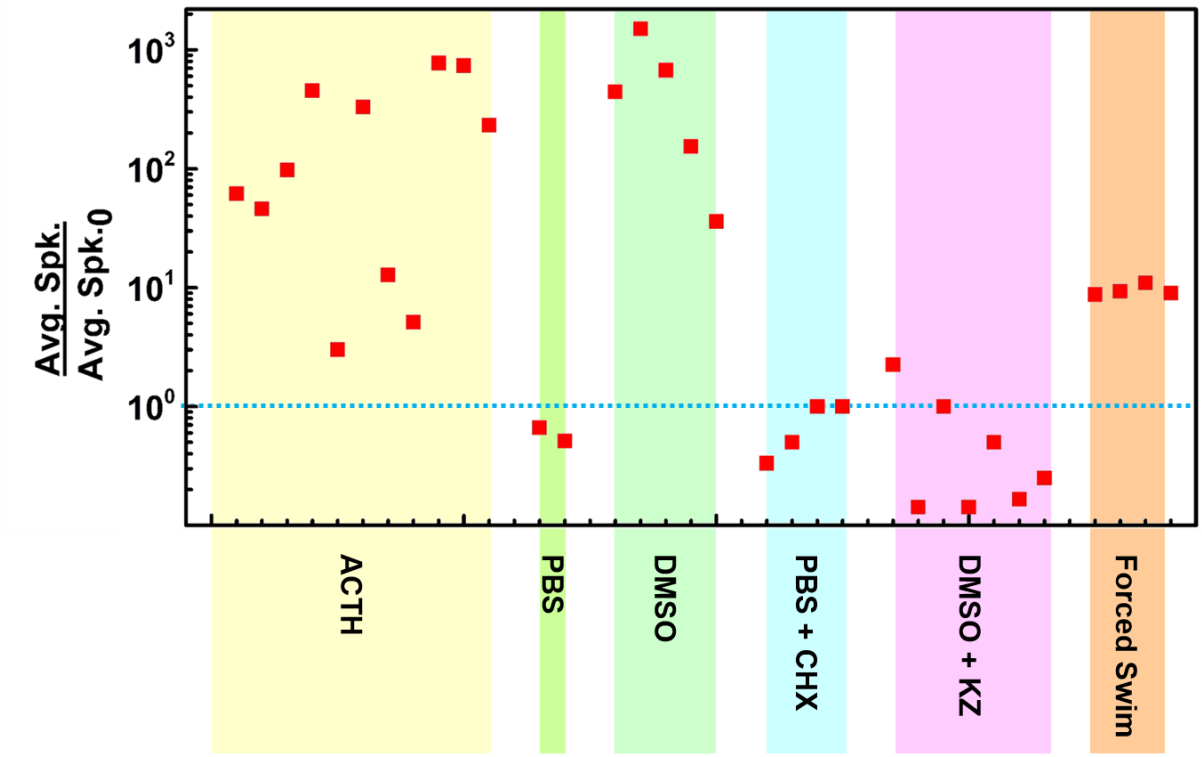

Fig. S9. Average spike ratio before and after injection or animal behavior test. Average spike frequency ratios before and after ACTH (11 rats), PBS (2 rats), DMSO (5 rats), DMSO+KZ (7 rats), PBS+CHX (4 rats), and forced swimming test (4 rats). The average spikes are obtained by 5 min recording. Red dots in each case show number of animals in different cases.

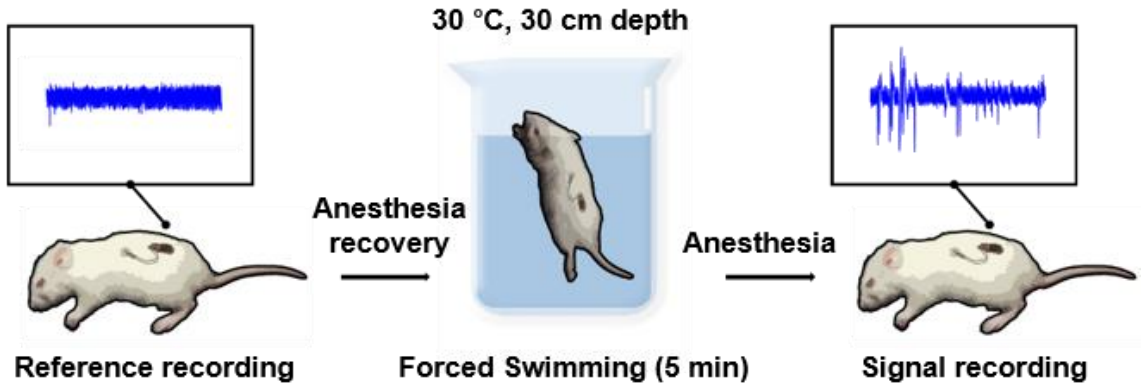

Fig. S10. Process scheme of the actual stress model. Rat recovered from anesthesia after short reference recording. Then the rat was gently placed in the water tank, and allowed to swim for 5 minutes. Five minutes later, the rat was rescued from the water, and quickly anesthetized. Signal change was then recorded.

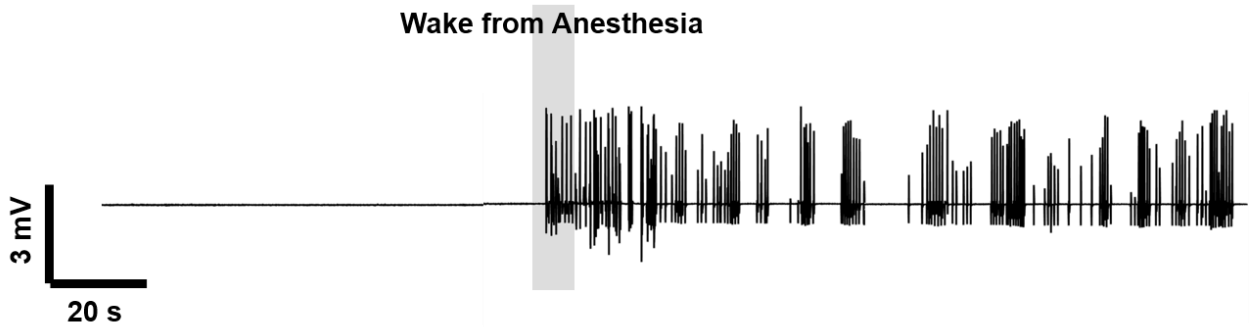

Fig. S11. Adrenal signal recording from freely-moving animal. The adrenal signal was collected from a rat that was weakly anesthetized with ketamine. As soon as the rat awoke from the anesthesia, stress was observed, due to the irritation and pain caused by the headstage and reference electrode.

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