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Lack of light-induced elevation of renal sympathetic nerve activity and plasma corticosterone levels in PACAP-deficient mice

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ABSTRACT

PACAP is a neurotransmitter involved in the signal transduction of light stimulation in the suprachiasmatic nucleus (SCN). Light stimulation affects autonomic nerve activity via the SCN, and here we tested whether PACAP participates in light-induced regulation of sympatho-adrenal activity by using PACAP-deficient (*Adcyap1*$^{-/-}$) mice. Light stimulation (100 lux, 30 min) significantly elevated both renal sympathetic nerve activity (RSNA), which was monitored on a digital oscilloscope, and plasma corticosterone levels in wild-type mice, but both responses were almost abolished in *Adcyap1*$^{-/-}$ mice. Although light-induced c-Fos expression in the SCN was observed in both genotypes, the numbers of c-Fos positive cells were significantly decreased in *Adcyap1*$^{-/-}$ mice. These data suggest that PACAP signaling pathway is involved in light-induced stimulation of RSNA and plasma corticosterone release through SCN of brain.

Light is a well-established cue for entraining circadian rhythm and directly and often immediately affects organism physiology [15]. These immediate effects, referred to as "masking", include pupillary light reflexes, suppression of plasma melatonin levels, elevation of sympathetic nerve activity and plasma corticosterone, reduction of heart rate, and suppression of behavioral activity in nocturnal animals [15,10,13]. The hypothalamic suprachiasmatic nucleus (SCN) is crucial for the generation and entrainment of circadian rhythm [15]. The SCN also regulates certain masking phenomena, including elevation of sympathetic nerve activity and plasma corticosterone levels, and reducing heart rate [10,13,14].

Pituitary adenylate cyclase-activating polypeptide (PACAP) is neuropeptide belonging to the vasoactive intestinal polypeptide (VIP)/glucagon/secretin family and exerts pleiotropic activities, including control of psychomotor and neurophysiological functions [18,9]. PACAP is expressed in a subset of the intrinsically photosensitive retinal ganglion cells (ipRGCs) innervating the SCN, and disappears following eye enucleation [7], suggesting that PACAP functions as a transmitter of light signals from the retina to the SCN. These ipRGCs are the principal regulators of light-induced entrainment of the circadian rhythm and several types of masking events [5]. The role of PACAP in photoentrainment of circadian rhythm were substantially examined [18,9,7], and recently evaluated in PACAP-deficient (*Adcyap1*$^{-/-}$) mice [11,3]. However, the role of PACAP in masking events is still unclear. By using *Adcyap1*$^{-/-}$ mice, here we examine the roles of endogenous PACAP in autonomic and humoral masking, such as the light-induced stimulation of renal sympathetic nerve activity (RSNA) and plasma corticosterone levels.

All animal experiments were performed in accordance with protocols approved by the Animal Care and Use Committee of the Graduate School of Pharmaceutical Sciences, Osaka University, and the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society. *Adcyap1*$^{-/-}$ mice [8] backcrossed onto a CD1 (ICR) mouse background at least 10 times were used. Mice were housed individually under a 12 h light/dark cycle (LD12:12) with food and water ad libitum for at

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least a week before experiments. In all experiments, mice were received a 30 min light pulse of 100 lux, since a 30 min light pulse of 20 lux, which induces a phase shift of the locomotor circadian rhythm in *Adcyap1*$^{-/-}$ mice [11], caused no significant changes in the renal sympathetic nerve activity (RSNA) or blood pressure (BP) in wild-type mice. RSNA and BP were measured as previously described [16]. The mice were fasted for 4–6 h prior to surgery and anesthetized with urethane (1 mg/kg, i.p.). A polyethylene catheter was inserted into the left femoral artery. For recording RSNA, the left nerve was exposed retroperitoneally through a left flank incision. The distal ends of the respective nerves were ligated and then attached to a pair of silver wire electrodes. The electrical signals from the electrodes were amplified, filtered, and monitored on an oscilloscope. The raw nerve activity was converted to standard pulses using a window discriminator. For recording blood pressure, a catheter in the left femoral artery was connected to a BP transducer whose output signal was amplified by a BP amplifier and averaged to obtain the mean arterial pressure (MAP). These signals were sampled with an analog-to-digital converter (PowerLab/4SP, AD Instruments, CA, USA), and stored on a hard disk drive for offline analysis as previously described [16]. In the electrophysiological study, mice were exposed to light illumination from zeitgeber time (ZT) 16.

For the analyses of plasma corticosterone levels and c-Fos expression in the SCN, mice were transferred to constant dark (DD) condition, and experiments were performed in the second DD cycle. Mice were received a 30 min light pulse of 100 lux from circadian time (CT) 16. Before the start of illumination (control), or 30 min, 60 min, or 90 min after the start of light exposure, mice were immediately decapitated under a dim red light and blood and brain samples were obtained. Blood was immediately centrifuged and plasma corticosterone levels were determined with the Rat Corticosterone ^{125}I Biotrack Assay System (GE Healthcare, Piscataway, NJ). Brains were excised, postfixed in 4% paraformaldehyde in phosphate-buffered saline at 4 °C overnight, and cryoprotected in 20% sucrose for two nights. The brains were then sliced using a cryomicrotome (CM1900, Leica, Heidenberg, Germany) in 20-μm thick coronal brain sections. Immunohistochemical analysis of c-Fos in the SCN was conducted as previously described [11]. Briefly, specific polyclonal rabbit antibodies against c-Fos (1:1000 dilution; Santa Cruz, South San Francisco, CA, USA) as primary antibodies and biotinylated anti-rabbit IgG as secondary antibodies (1:500 dilution; Vector Laboratories, Burlingame, CA, USA) were used for immunostaining. The immunoreactivity was visualized with the Vectastain ABC kit (Vector Laboratories, CA, USA) and diaminobenzidine (Sigma, MO, USA) as the chromogen. To determine the area of the SCN, adjacent sections were stained by 4′,6-diamino-2-phenylindole (DAPI) and the SCN was delineated. Slices (5–8 slices per brain) were photographed under a microscope (BX51, Olympus, Tokyo, Japan), and the number of c-Fos immunoreactive (IR) nuclei and DAPI-stained nuclei in the SCN was counted using Photoshop (Adobe Software, CA, USA). For each brain, estimated counts from 5 to 8 sections per SCN were summed to determine a final cell count of c-Fos-IR nuclei. One analysis used a 9 × 7 array of squares (Fig. 3C), each square being a 50 μm × 50 μm template, placed to encompass most of the SCN plus the adjacent hypothalamus. The same slices used to count c-Fos-IR nuclei were analyzed on a per square basis to examine the dorso-ventral distribution of c-Fos-IR nuclei. For each brain, the number of c-Fos-IR nuclei in each square was averaged over 5–8 brain sections to determine the average c-Fos-IR number in each area. Statistical analysis was performed with Statview software.

We first examined light-induced sympathoexcitation in *Adcyap1*$^{-/-}$ mice and wild-type controls under anesthetized conditions. Fig. 1 shows the effects of light stimulation to the eyes on blood pressure (BP) and the efferent activity of the renal branch of the splanchnic nerve. Light illumination caused a gradual and robust increase in the discharge rate of the renal nerve in wild-type mice, but not in *Adcyap1*$^{-/-}$ mice (RSNA at 90 min vs. the baseline level, *Adcyap1*$^{-/-}$ mice, 84 ± 15%; wild-type mice, 230 ± 32%) (Fig. 1A). Although these differences were significant (Fig. 1B, left panel), there was no difference in basal levels of RSNA ($p > 0.1$, Mann–Whitney *U*-test). Basal levels of BP were not significantly different between the two genotypes ($p > 0.1$, Mann–Whitney *U*-test), and light exposure did not affect BP in both genotypes in the present experimental conditions (Fig. 1B, right panel).

We next examined the light-induced elevation of plasma corticosterone levels, another masking phenomenon, in both genotypes. In wild-type mice, light stimulation significantly increased plasma corticosterone levels, with a transient peak of approximately twofold above basal at 60 min after light exposure. However, this effect was not seen in *Adcyap1*$^{-/-}$ mice (Fig. 2). Adrenal sympathetic denervation also blocks this transient increase in corticosterone [10], suggesting that light-induced sympatho-adrenal excitation is

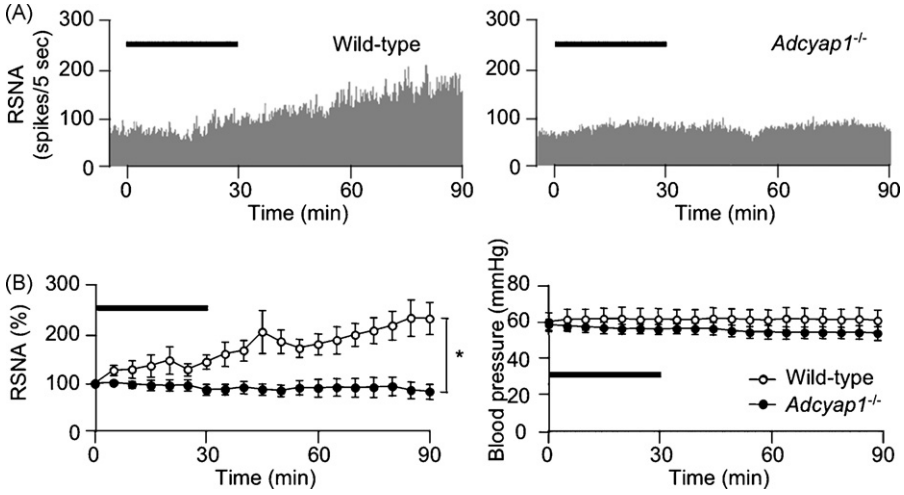

Fig. 1. Effects of light exposure on the sympathetic nerve activity of kidney and blood pressure in urethane-anesthetized *Adcyap1*$^{-/-}$ mice. Mice were exposed to light (100 lux, 30 min) from ZT16 as indicated by the bold lines. (A) Representative trace data from recordings of renal sympathetic nerve activity (RSNA) before and after the light exposure in *Adcyap1*$^{-/-}$ and wild-type mice. (B) Changes in RSNA (left panel) and blood pressure (right panel) in *Adcyap1*$^{-/-}$ and wild-type mice after the light exposure. Data are expressed as mean ± S.E.M. ($n = 5$–6). Relative changes in RSNA are presented as percentages of the values at 0 min. *$p < 0.05$, repeated two-way ANOVA.