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# IL-6 knockout mice are protected from cocaine-induced kindling behaviors; possible involvement of JAK2/STAT3 and PACAP signalings

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ABSTRACT

IL-6 has been recognized as an anticonvulsant against certain neuroexcitotoxicities. We aimed to investigate on the interactive role between IL-6 and PACAP in cocaine-induced kindling behaviors. Although we found that cocaine (45 mg/kg, i.p./day x 5) significantly increased IL-6 and TNF-α expression, it resulted in a decrease in IFN-γ expression. We observed that the cocaine-induced increase in IL-6 expression was more pronounced than that in TNF-α expression. Genetic depletion of IL-6 significantly activated cocaine kindling behaviors. This phenomenon was also consistently observed in WT mice that received a neutralizing IL-6 receptor antibody. Cocaine-treated IL-6 knockout mice exhibited significantly decreased PACAP and PACAP receptor (PAC1R) mRNA levels and significantly increased TNF-α gene expression. TNF-α knockout mice were protected from cocaine kindling via an up-regulation of IL-6, phospho-JAK2/STAT3, PACAP, and PAC1R levels, which produced anti-apoptotic effects. Recombinant IL-6 protein (rIL-6, 2 μg, i.v./mouse/day x 5) also up-regulated phospho-JAK2/STAT3, PACAP, and PAC1R mRNA levels, leading to anti-apoptotic effects in IL-6 knockout mice. Consistently, AG490, a JAK2/STAT3 inhibitor, and PACAP 6–38, a PAC1 receptor antagonist, counteracted rIL-6-mediated protection. Combined, our results suggest that IL-6 gene requires up-regulation of phospho-JAK2/STAT3, PACAP, and PAC1R and down-regulation of the TNF-α gene to modulate its anticonvulsive/neuroprotective potential.

## 1. Introduction

It is recognized that convulsions begin to occur when initially sub-convulsive doses are injected repeatedly (Davis, 1996). Earlier studies have indicated that repetitive exposure of sub-convulsive doses of cocaine is also associated with an increased risk of convulsive behaviors in rodents and monkeys (Miller et al., 2000; Post and Kopanda, 1975, 1976; Post and Rose, 1976; Stripling and Ellinwood, 1977; Tatum and Seevers, 1929). Repeated administration of sub-convulsive doses of cocaine can increase sensitivity to its convulsant effect, a phenomenon called *kindling* that is analogous to the kindling of epileptic seizures engendered by repetitive sub-threshold electrical stimulation of the limbic system (Goddard et al., 1969). Cocaine-kindling behaviors have been recognized as an advantageous model for studying the psychopathology and toxicity associated with cocaine abuse (Miller et al., 2000). Indeed, prolonged cocaine abuse is not only associated with increases in seizure probability (Alldredge et al., 1989; Dhuna et al., 1991), but also with violent behaviors (Richards et al., 1998; Ruttenber

*Abbreviations:* GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL-6, interleukin-6; IFN-γ, interferon- γ; JAK2, janus-activated kinase 2; KO, knockout; mRNA, messenger ribonucleic acid; PACAP, pituitary adenylate cyclase-activating polypeptide; PAC1R, pituitary adenylate cyclase-activating polypeptide receptor 1; PI3K, phosphoinositide 3-kinase; PTN, pleiotrophin; rIL-6, recombinant IL-6; RT-PCR, reverse transcription–polymerase chain reaction; STAT3, signal transducer and activator of transcription 3; TMT, trimethyltin; TNF, tumor necrosis factor; Veh, vehicle; WT, wild type

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et al., 1997) and panic disorders (Louie et al., 1989). Overlapping pharmacological mechanisms have been proposed to elucidate those neurological and behavioral disorders (Post, 2002).

Neuroprotective and adaptive responses to cocaine-induced neurotoxicity and neurodegenerative processes include cerebral up-regulation of neurotrophic cytokines, including pleiotrophin (PTN) and midkine (Vicente-Rodriguez et al., 2013, 2015). Some of the signaling pathways triggered by PTN are important in the neuroprotective roles of those cytokines against cocaine-induced neurotoxicity (Herradon and Perez-Garcia, 2014). For example, PTN has been shown to prevent cocaine-induced cytotoxicity in NG108-15 and PC12 cell cultures (Gramage et al., 2008; Herradon et al., 2009). In addition, evidence points to a modulatory role for PTN in inflammation. PTN induced the expression of various cytokines, including tumor necrosis factor (TNF-α) and interleukin (IL)-6, in peripheral organs (Achour et al., 2008). TNF-α and IL-6 levels increased significantly in response to lipopolysaccharide in PTN-transgenic mice (Fernandez-Calle et al., 2017).

Increasing evidence suggests that cytokines participate not only in functions related to the immune system, but also in complex functions of the central nervous system, such as seizures (Dey et al., 2016; Li et al., 2011). In particular, IL-6 is reported to modulate seizure activity (Alapirtti et al., 2018; Ambrogini et al., 2018; Li et al., 2011). We previously demonstrated that IL-6 protects against trimethyltin (TMT)-induced excitotoxicity via an Nrf-2-dependent glutathione defense mechanism and phosphoinositide 3-kinase (PI3K)/Akt-dependent signaling (Tran et al., 2012). We also observed that recombinant IL-6 protein (rIL-6) significantly inhibited memory impairments induced by TMT in IL-6 knockout (KO) mice (Kim et al., 2013). The administration of pituitary adenylate cyclase-activating polypeptide (PACAP) to wild-type (WT) mice significantly increased IL-6 level in the cerebrospinal fluid and IL-6 mRNA expression in the brain, indicating that IL-6 is an important factor mediating the neuroprotective effect of PACAP in response to brain ischemia (Ohtaki et al., 2006).

The neuroprotective activities of endogenous PACAP have been evaluated using PACAP KO mice, which exhibit worse neurologic symptoms after middle cerebral artery occlusion than WT mice (Nakamachi et al., 2010; Reglodi et al., 2012). In addition, PACAP protects nerve cells through the inhibition of cytochrome C release from the mitochondria (Ohtaki et al., 2006), and promoted the secretion of IL-6 from astrocytes and Müller cells (Nakatani et al., 2006; Tatsuno et al., 1996). IL-6-induced up-regulation of growth factors known to act in the induction of PACAP, which could have an important effect on IL-6-induced PC12 cell differentiation (Ravni et al., 2006). Microarray analyses of PACAP-regulated gene transcripts revealed that many gene families that are activated by PACAP in primary sympathetic neurons were also induced by IL-6 in PC12 cells (Braas et al., 2007). Therefore, it is plausible that the effects of IL-6 are mediated by the intermediate action of PACAP.

The neuroprotective properties of PACAP have been established in ischemic neuronal injuries (Ohtaki et al., 2006; Reglodi et al., 2018; Shioda and Nakamachi, 2015). Lower levels of phosphorylated STAT3 and phosphorylated ERK were observed in PACAP ± mice, whereas a reduction in phosphorylated STAT3 was recorded in IL-6 KO mice, suggesting that PACAP prevents neuronal cell death after ischemia via a signaling mechanism involving IL-6 and STAT3 (Ohtaki et al., 2006). Because IL-6 also modulates convulsive behaviors (Tran et al., 2012; Tu et al., 2017), we designed the present study to explore the roles of IL-6 and PACAP in the expression and development of cocaine-kindling behaviors. We propose that PACAP is an important modulator of IL-6-mediated anticonvulsant protection against cocaine insult.

## 2. Materials and methods

### 2.1. Animals

All mice were treated in strict accordance with the NIH Guide for the Humane Care and Use of Laboratory Animals. Eight-week-old male C57BL/6 (WT) mice, male IL-6 KO mice, and male TNF-α KO mice weighing 25 ± 3 g were used for this study. WT animals were purchased from Orient Bio Inc. (Charles River Technology, Seoul, Korea). We used IL-6 KO and TNF-α KO mice of the C57BL/6 background strain, as previously reported (Clark et al., 2000; Hoshi et al., 2009; Kim et al., 2013; Sudo et al., 2008; Tran et al., 2012). Breeding pairs were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Mice were bred and housed in an approved animal facility at Kangwon National University. They were maintained on a 12/12-h light/dark cycle and fed ad libitum. They were adapted to these conditions for 2 weeks before the experiment.

### 2.2. Drug treatment and experimental design

Cocaine hydrochloride, IL-6 receptor neutralizing monoclonal antibody (IL-6R Ab; Chugai Pharmaceutical Co., Ltd., Tokyo, Japan), rIL-6 protein (BD Pharmingen, San Diego, CA, USA), TNF-α neutralizing monoclonal antibody (TNF-α Ab; R&D System, Minneapolis, MN, USA), and PACAP potent competitive receptor PAC1 antagonist (PACAP 6–38; a gift from professor Dora Reglodi - Department of Anatomy, MTA-PTE PACAP Research Group, University of Pecs Medical School, Pecs, Hungary) were dissolved in sterile saline immediately before use. AG490 (Tocris Bioscience, Ellisville, MO, U.S.A.), an inhibitor of JAK2/STAT3, was dissolved in 50% dimethyl sulfoxide (DMSO) in sterile saline (Chiba et al., 2009; Jung et al., 2009; Tran et al., 2016) immediately before use.

IL-6 KO mice received rIL-6 (2 μg, i.v./mouse/day x 5) 1 h before every cocaine treatment (45 mg/kg, i.p./day x 5). WT mice and IL-6 KO mice (with or without rIL6) were euthanized 1 h, 6 h, 12 h, 1 d, 3 d, 7 d, or 14 d post-cocaine (Supplementary Fig. 1A). To confirm the role of cytokines, TNF-α KO mice received cocaine (45 mg/kg, i.p./day x 5) and were euthanized 12 h post-cocaine (Supplementary Fig. 1A). To clarify the roles of the IL-6 gene, JAK2/STAT3, and PACAP, mice received AG490 (5 mg/kg or 10 mg/kg, i.p./day x 5), IL-6R Ab (1 mg/kg, i.p./day x 5), rIL-6 (2 μg/i.v./day x 5), or TNF-α Ab (1 mg/kg, i.p./day x 5) 1 h prior to cocaine. Control mice received vehicle (50% DMSO in sterile saline; 1 ml/kg, i.p./day x 5) (Supplementary Fig. 1B). PACAP 6–38 (6 μg, i.v./mouse/day or 60 μg, i.v./mouse/day) was injected 1 h after cocaine treatment, and mice were euthanized 12 h post-cocaine (Supplementary Fig. 1C). Mice were euthanized by decapitation, and hippocampus was immediately dissected and stored in liquid nitrogen for further analyses. Doses and other experimental conditions were determined based on previous studies (Ohtaki et al., 2008, 2010; Wang and Dunn, 1998) and our pilot study (Mai et al., 2016).

### 2.3. Assessment of cocaine-induced kindling behaviors

To assess the kindling behaviors induced by cocaine, mice were placed separately in Plexiglas containers (14 × 25 × 36 cm high) for observation. The presence or absence of convulsions was recorded 30 min following injection. The definition of a cocaine convulsion was the loss of the righting response for at least 5 s and the occurrence of clonic limb movements (characterized by rapid rhythmic contraction and relaxation of muscles in extremities or episodes of violent and dramatic uninhibited running and jumping/bouncing). Kindling was defined as an increase in the percentage of mice exhibiting seizures with repeated exposure to cocaine. The seizure score was generated using the following weighted formula based on latencies to the three different components of the seizure phenotype: Seizure score = [(latency to forelimb or hindlimb clonus)$^{-1}$ x 1000 × 0.2] + [(latency to clonic running seizure) $^{-1}$ x 1000 × 0.3] + [(latency to clonic jumping/bouncing seizure)$^{-1}$ x 1000 × 0.5]; where 0.2, 0.3, and 0.5 are the weights of the respective components. The phenotype components occur in a consistent progression from clonus to jumping/bouncing seizures in mice. The total seizure score was calculated as the sum