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Brain region-specific reduction in c-Fos expression associated with an anxiolytic effect of yokukansan in rats

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ABSTRACT

Ethnopharmacological relevance: A traditional Japanese (Kampo) medicine, yokukansan, has long been used to treat neurosis, insomnia, and night crying and irritability in children. Recently, this medicine has reported to improve the behavioral and psychological symptoms of dementia that often become problematic in patients with Alzheimer's disease and other forms of dementia.
Aim of the study: Several animal studies have reported that yokukansan has an anxiolytic effect. However, the underlying mechanisms are not yet understood. In the present study, we investigated the effects in rats of single and repeated administrations of yokukansan on anxiety-like behaviors, stress responses, and the brain regions involved.
Materials and methods: Yokukansan dissolved in water (100 or 300 mg/kg) was administered orally to F344/N male rats 1 h before each test or for two weeks before the tests began. Locomotor activity and anxiety-related behavior in the open-field test and the elevated plus-maze test, serum corticosterone levels, and restraint stress-induced c-Fos expression in various brain regions as a marker of neuronal activation were evaluated in both the vehicle-treated and yokukansan-treated rats.
Results: A single administration of yokukansan had no effect on locomotor activity or anxiety-like behavior; however, repeated administration decreased anxiety-like behavior in a dose-dependent manner. Neither single nor repeated administration of yokukansan had an effect on the basal or stress-induced levels of serum corticosterone. For c-Fos expression, restraint stress increased the number of c-Fos-positive cells in the subdivisions of the prefrontal cortex, amygdala, and hypothalamus. Repeated administration of yokukansan decreased the stress-induced c-Fos expression in the prelimbic cortex and the basolateral and medial amygdaloid nuclei.
Conclusions: The present study indicates that repeated oral administration of yokukansan has an anxiolytic effect and that this effect may be associated with attenuated neuronal activity in the medial prefrontal cortex and amygdala.

1. Introduction

In humans, anxiety is a common physiological and psychological response to stressors. Excessive and accumulated stress is thought to be a risk factor for psychiatric disorders, including anxiety disorder and major depression (Anisman and Farabollini, 1982; Mazure, 1995). Individuals suffering the psychiatric disorders also experience intense and prolonged anxiety in daily life situations. Stressors activate the hypothalamic–pituitary–adrenal (HPA) axis, resulting in the release of glucocorticoid hormones (cortisol or corticosterone) that enhance an organism's ability to adapt to stress (Sapolsky et al., 1986; Porter et al., 2001). It is known that psychiatric disorders are associated with abnormal HPA axis activity, which results in hypersecretion of glucocorticoids and diminished glucocorticoid negative feedback (Carroll et al., 1981; Kalin et al., 1982; Holsboer, 1983; Arana et al., 1985). Long-lasting exposure to glucocorticoids can induce dysfunction in the negative feedback loop and can increase anxiety- and depression-like behaviors (Gregus et al., 2005; Ardayfio and Kim, 2006; Johnson et al., 2006; Mitra and Sapolsky, 2008). Thus, anxiety and HPA responses to stress are of great interest to those seeking to understand the biological mechanisms underlying psychiatric disorders as well as to researchers working to develop therapeutic drugs.

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Many psychopharmacological studies have reported that several substrates, including $GABA_A$-benzodiazepine receptor and serotonergic receptor ligands, are involved in the control of anxiety and fear in humans and rodents (Hoehn-Saric, 1982; Hardman et al., 2001), and therefore, these substrates have been used frequently to treat emotional disturbances in several neuropsychiatric disorders. On the other hand, crude drugs extracted from natural plants have also been used to treat neurodegenerative and psychiatric disorders because of their efficacy and their relative safety (Kanba et al., 1998). In Japan, yokukansan, a traditional Japanese herbal medicine (also called Kampo medicine), has attracted interest for its efficacy in patients with neurodegenerative disorders. For example, 4-week treatment with yokukansan has been reported to ameliorate behavioral and psychological symptoms of dementia (BPSD), including delusions, hallucinations, agitation/aggression, depression, anxiety, and irritability, in Alzheimer's disease and several other types of dementia (Iwasaki et al., 2005; Mizukami et al., 2009; Monji et al., 2009). These findings suggest that yokukansan may have a wide variety of therapeutic effects in mental illness.

Yokukansan is a hot water-extracted dried powder that is composed of a mixture of seven medicinal herbs (see Section 2). It has been approved by the Ministry of Health, Labour and Welfare of Japan as a remedy for neurosis, insomnia, and irritability and night crying in children. To investigate the possible effects of yokukansan on brain function, a number of behavioral studies have been conducted in rodents. For example, repeated administration for 14 days of yokukansan improved aggressive behaviors in rats that were treated with the serotonergic neurotoxin p-chloroamphetamine (Kanno et al., 2009). This drug also ameliorated cognitive dysfunction and anxiety-like behaviors as well as aggressive behaviors in zinc-deficient rats (Takeda et al., 2008a; 2008b), olfactory bulbectomized mice (Yamada et al., 2011), rats subjected to repeated cerebral ischemia (Nogami et al., 2011), and transgenic mice overexpressing amyloid precursor protein (APP-Tg mice) (Tabuchi et al., 2009; Fujiwara et al., 2011), which are frequently used as animal models for psychiatric disorders, cerebrovascular dementia, and Alzheimer's disease. Thus, although it has been reported that yokukansan has ameliorative effects on several behavioral deficits, its precise effects on behavior as well as on the neuroendocrine response of the HPA axis to stress and the underlying brain mechanisms remain to be elucidated.

In the present study, to determine the effects of single and repeated administration of yokukansan on anxiety and the HPA system, we investigated locomotor activity and anxiety-like behavior in a novel environment and serum corticosterone levels in response to restraint stress in vehicle- and drug-treated male F344/N rats. We found that repeated administration of the drug for 2 weeks had an anxiolytic effect. To identify the possible brain mechanisms involved in this anxiolytic effect, we further examined stress-induced c-Fos expression as a marker of neuronal activation (Kovács, 1998) in brain regions that are related to neural circuits associated with anxiety and the stress response (Kalisch et al., 2004; Salomé et al., 2004; Hale et al., 2006; Bouwknecht et al., 2007; Shoji and Mizoguchi, 2010) in these rats.

2. Materials and methods

2.1. Animals

F344/N male rats (Japan SLC, Inc., Shizuoka, Japan), 13–15 weeks of age, were used ($n=81$ in total). The animals were housed two per plastic cage ($25 \times 41 \times 20$ cm), which had a stainless-steel lid. Water and food were available ad libitum. The laboratory was maintained on a 12-h light/dark cycle (lights on at 08:00) at 23 ± 1 °C with 55 ± 5% humidity. All procedures were approved by the Experimental Animal Committee of the National Center of Geriatrics and Gerontology and complied with the Guidelines for Animal Experimentation of the committee.

2.2. Drug

Yokukansan in the form of a dried powder was supplied by Tsumura & Co. (Tokyo, Japan). It consists of seven dried medicinal herbs; Atractylodes Lancea rhizome (4.0 g, rhizome of *Atractylodes lancea* De Candolle), Poria sclerotium (4.0 g, sclerotium of *Poria cocos* Wolf), Cnidium rhizome (3.0 g, rhizome of *Cnidium officinale* Makino), Uncaria Hook (3.0 g, thorn of *Uncaria rhynchophylla* Miquel), Japanese Angelica root (3.0 g, root of *Angelica acutiloba* Kitagawa), Bupleurum root (2.0 g, root of *Bupleurum falcatum* Linné) and Glycyrrhiza (1.5 g, root and stolon of *Glycyrrhiza uralensis* Fisher). Each plant material was identified by its external morphology and authenticated by marker compounds of plant specimens according to the methods of the Japanese Pharmacopoeia and the standard of Tsumura & Co. The seven medical herbs were mixed, and extracted with purified water at 95 °C for 1 h, and the extraction solution was separated from the insoluble waste and concentrated by removing water under reduced pressure. Spray-drying was used to produce a dried extract powder. The yield of the extract was approximate 15.9%. The quality was standardized based on the Good Manufacturing Practice defined by the Ministry of Health, Labour and Welfare of Japan. Moreover, the constituents of the yokukansan extract have been defined by a three-dimensional high performance liquid chromatographic analysis (Mizukami et al., 2009).

Yokukansan was dissolved in distilled water (vehicle), and it or vehicle was orally administered to rats (100 or 300 mg/kg) in a dose volume of 10 ml/kg using a stainless sonde and a syringe.

2.3. General procedure

In the single-administration experiment (Fig. 1A), rats ($n=40$) were assigned to either vehicle group ($n=14$), yokukansan (100 mg/kg/day) group ($n=12$), or yokukansan (300 mg/kg/day) group ($n=14$). Each group was left undisturbed until the beginning of the behavioral tests. On test day 1, each group was subjected to the open field test 1 h after the drug administration, and on test day 2, they were tested in the elevated plus maze 1 h after the drug administration. On test day 3, after the drug administration, each group was either left undisturbed or exposed to 60 min-restraint stress (vehicle, $n=6$, 8; 100 mg/kg yokukansan, $n=6$, 6; 300 mg/kg yokukansan, $n=6$, 8, respectively), and then rats were sacrificed to collect blood and to assess serum corticosterone level (see details below). In the open field test, one out of 14 vehicle-administrated rats was excluded from the analysis, because the rat's behavior was interrupted by unexpected matters.

In the repeated-administration experiment (Fig. 1B), rats ($n=41$) were daily administrated to either vehicle ($n=16$), 100 mg/kg yokukansan ($n=12$), or 300 mg/kg yokukansan ($n=13$) for 14 days. On test day 15, 1 h after the drug administration, each group was subjected to the open field test, and on the following day, they were tested in the elevated plus maze 1 h after the drug administration. Following the last administration, each group was either left undisturbed or exposed to 60 min-restraint stress (vehicle, $n=8$, 8; 100 mg/kg yokukansan, $n=6$, 6; 300 mg/kg yokukansan, $n=6$, 7, respectively), and thereafter, rats were sacrificed to collect blood and brain and to assess serum corticosterone level and c-Fos expressions in the brain (see details below).