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Evidence for a role of inhibition of orexinergic neurons in the anxiolytic and sedative effects of diazepam: A c-Fos study

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ABSTRACT

The classical benzodiazepine diazepam (DZ) induces anxiolysis at low doses and sedation and hypnosis at higher doses. Different brain areas and neuronal populations most likely mediate these different behavioral effects. We used c-Fos immunohistochemistry as an indirect way to study neuronal activation or inhibition induced by DZ at anxiolytic and sedative doses (0.5 and 5 mg/kg, respectively) in various brain areas involved in anxiety, arousal, sedation and addiction in C57BL/6J mice. We also focused on the two neuronal populations, orexinergic and dopaminergic neuronal populations, with the help of double-immunohistochemistry using c-Fos and orexin-A antibodies and c-Fos and tyrosine hydroxylase antibodies. We found that different brain areas of unhabituated mice reacted differently to the mild stress induced by vehicle injection. Also the response to anxiolytic or sedative doses of DZ differed between the areas, suggesting that distinct brain areas mediate the behavioral effects of low and high DZ doses. Our findings propose a role for inhibition of orexin neurons in the anxiolytic and sleep-promoting effects of DZ. In addition, the activation of central amygdala neurons by DZ treatment was associated with anxiolytic and sedative effects. On the other hand, the ventral hippocampus, basolateral amygdala, ventral tegmental area and prefrontal cortex were sensitive even to the mild injection stress, but not to the anxiolytic dose of DZ.

1. Introduction

Benzodiazepine (BZ) agonists are positive allosteric modulators of γ-aminobutyric acid type A ($GABA_A$) receptors. They dose-dependently induce anxiolytic, muscle relaxant, anticonvulsant, sedative and hypnotic effects (Korpi and Sinkkonen, 2006). Different point mutations in $GABA_A$ receptor subunits rendering receptors insensitive to BZs have provided a powerful tool to dissect the subunit compositions and brain areas important in different behavioral features induced by BZs in mice (see for review, Rudolph and Möhler, 2004). $GABA_A$ receptors containing the α1 subunit seem important for sedation, anterograde amnesia and anticonvulsant actions of BZs (Rudolph et al., 1999).Surprisingly, the BZ effects on sleep latency, amount of sleep and on some specific features of sleep are not affected or are even enhanced in the mice with BZ-insensitive α1 subunits, whereas in mice with BZ-insensitive α2 subunits the effect of BZs on sleep is reduced suggesting an important role for α2 subunit-containing $GABA_A$ receptors in the hypnotic effects of BZs (Kopp et al., 2004; Tobler et al., 2001; Winsky-Sommerer, 2009). The α2 subunit was found important also for the anxiolytic as well as the myorelaxant actions of BZs whereas the α3 subunit seemed to reduce the myorelaxant action of high doses of BZs (Crestani et al., 2001; Löw et al., 2000). The distribution of subunits fits well with these findings, because α1 subunit-containing receptors are expressed widely in the brain, whereas α 2 is expressed mostly in sleep–wake regulating hypothalamus and in the limbic regions important for emotional stimuli processing (Fritschy and Mohler, 1995).

In contrast to molecular mechanisms, a consensus about the brain areas and neuronal mechanisms behind the therapeutic and adverse effects of BZs has not been reached. Instead of inducing sedation and sleep simply by suppressing activity widely across the brain, BZs might affect specific target areas that regulate arousal state and sleeping and waking phases. As an example, the hypothalamus seems to be a key regulator of sleep and waking and has been suggested to mediate the sedative and hypnotic actions of GABA mimetic drugs like BZs (Lu and Greco, 2006; Nelson et al., 2002; Zecharia et al., 2009). BZs binding to synaptic (especially α1 and α2 subunit-containing) $GABA_A$ receptors might preferentially inhibit the wake-active histamine and orexin (ORX, also known as hypocretin)

Abbreviations: ANOVA, one-way analysis of variance; BLA, basolateral nucleus of amygdala; BSA, bovine serum albumin; BZ, benzodiazepine; CA1, CA1 pyramidal cell layer of the hippocampus; CA3, CA3 pyramidal cell layer of the hippocampus; CeL, lateral division of the central nucleus of amygdala; CeM, medial division of the central nucleus of amygdala; CPu, caudate-putamen; CRF, corticotrophin-releasing factor; DG, granular cell layer of dentate gyrus; DM-PFA, dorsomedial hypothalamic nucleus/perifornical area; DA, dopamine; DZ, diazepam; EPM, elevated plus-maze; $GABA_A$, γ-aminobutyric acid type A; IEG, immediate early gene; LH, lateral hypothalamus; mPFC, medial prefrontal cortex; NAc, nucleus accumbens; ORX, orexin; RT, room temperature; SNC, substantia nigra compacta; TBST, tris-buffered saline supplemented with 0.05% Tween 20; TH, tyrosine hydroxylase; VTA, ventral tegmental area.

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neurons in the hypothalamus thus reducing arousal and promoting sleep, and as a different example, the GABA-site agonist gaboxadol (THIP), targeting extrasynaptic α4δ subunit-containing $GABA_A$ receptors, preferentially activates the sleep-active GABAergic neurons in the ventrolateral preoptic nucleus thereby inducing sleep (Lu and Greco, 2006).

Also many theories about the mechanisms behind the undesired effects of BZs have been proposed. Hyperexcitability of the brain and withdrawal anxiety and insomnia might develop as homeostatic adaptations to strong depression of brain activity by BZ treatment, and be manifested when e.g. glutamatergic neurotransmission is heightened at the disappearance of BZs from the brain (Izzo et al., 2001; Shen et al., 2010; Stephens, 1995). On the other hand, BZs seem to affect the dopamine (DA) pathways arising from the ventral tegmental area (VTA) in a similar way as other drugs of abuse. BZs activate the DA neurons in the VTA by inhibiting the inhibitory transmission coming to them from the GABAergic interneurons, and this leads to glutamatergic plasticity in DA neurons which is thought to represent an early step in the development of addictive behavior (Heikkinen et al., 2009; O'Brien and White, 1987; Tan et al., 2010).

In order to understand in more detail the neuronal populations and brain regions affected by BZs in young and adult mice, we have studied the effect of the classical BZ agonist diazepam (DZ) on neuronal activity indirectly by using c-Fos immunohistochemistry. Expression of the immediate early gene c-Fos is induced in cells whose membrane is depolarized e.g. during neuronal activity (Hughes and Dragunow, 1995). We studied two neuronal populations more carefully: the DA neurons in the midbrain and the ORX neurons in the hypothalamus. The c-Fos & ORX-A and c-Fos & tyrosine hydroxylase (TH)-double labeling was used to enable the recognition of these neuronal populations specifically. The VTA DA neurons were of interest for us because of our earlier studies examining the effect of BZs on them, and for their possible role in BZ reinforcement (Heikkinen et al., 2009). In addition DAergic transmission arising from the VTA and substantia nigra compacta (SNC) is important for arousal and anxiety state of an animal, and in the regulation of motivation and voluntary motor movements (Chinta and Andersen, 2005). Nucleus accumbens (NAc) shell and core regions, prefrontal cortex (PFC) and caudate-putamen (CPu), the targets of these DAergic projections were also studied. It has been suggested that DAergic activity in striatum mediates the locomotor-activating effects of low BZ doses (Bentue-Ferrer et al., 2001; Soderpalm et al., 1991). Other areas of interest were the dorsal and caudal/ventral hippocampus and the amygdala. The dorsal and ventral portions of hippocampus have different connectivity with different brain regions and both behavioral and gene expression data support the division of the hippocampus into separate regions where the dorsal part performs primarily cognitive functions and the ventral part relates to stress, emotion and affect (see for review, Fanselow and Dong, 2010). Amygdala is an area involved in the regulation of not only stress and fear-conditioning, but also addiction. Amygdala has often been suggested to mediate the effects of anxiolytic drugs, such as BZs (File, 2000; Killcross et al., 1997), but also other brain areas such as the hippocampus or lateral septum have been argued as essential in mediating the BZ anxiolysis (Treit and Menard, 1997).

ORXs are neuropeptides synthesized by neurons in the lateral hypothalamus (LH) and dorsomedial-perifornical hypothalamic areas (DM-PFA) that promote arousal and regulate the transitions between sleep and wake (Beuckmann and Yanagisawa, 2002). Antagonists of ORX receptors dose-dependently reduce locomotor activity and promote sleep (Winrow et al., 2011). On the other hand, ORXs have been found to be important in reward-seeking and addiction (Aston-Jones et al., 2010). Thus, ORXs interact with various brain mechanisms regulating emotion, reward and energy homeostasis to set the right arousal level for consummatory and protective behaviors, such as finding food and avoiding danger (Sakurai and Mieda, 2011). ORXs are also involved in states of high-arousal, such as stress and drug seeking (Berridge et al., 2010; Harris et al., 2005). In addition, they orchestrate neural circuits that control autonomic functions related to emotional behaviors such as respiration, blood pressure and stress-induced analgesia (Kuwaki, 2011). ORXs are involved in the pathophysiology of panic anxiety: patients with panic anxiety have elevated levels of ORX in cerebrospinal fluid, decreasing GABA in DM-PFA produces anxiety-like states in rats, and activation of ORX neurons is necessary for panic-prone state (Johnson et al., 2010). BZs have been shown to offer rapid and effective relief of panic anxiety symptoms (see for review, Ravindran and Stein, 2010). Thus, ORX neurons may be involved in a number of behaviors that BZs are used to alleviate, making it possible that BZs interact with the hypothalamic ORX system.

2. Material and methods

2.1. Animals

C57BL/6J mice were purchased from Charles River (Charles River Laboratories GmbH, Sulzfeld, Germany) and maintained for up to three generations at our facility. Male mice either at the age of 21–30 days (young) or 10–13 weeks (adult) were used in the experiments. Young mice were weaned 2–3 days before the experiments. The first c-Fos experiment was done with DZ at the dose of 5 mg/kg in young mice (21–30 days), because we had used this dosing in young mice in previous electrophysiological studies (Heikkinen et al., 2009). Brains were collected for c-Fos labeling at three different time-points after the injection to catch 1) the first acute effect (2 h, to allow time for c-Fos protein to build up or degrade after neuronal activation or deactivation, respectively), 2) the point when the animals are recovering from sedation (5 h), and 3) the time-point when we had earlier found the long-lasting modulations of glutamatergic transmission in VTA DA neurons (24 h). After our first findings in young mice, we extended the study to adult mice, and to two different doses of DZ (anxiolytic 0.5 mg/kg and sedative 5 mg/kg) (Garrett et al., 1998).

Mice were group-housed (2–5 animals per cage) in standard housing conditions (12-h light–dark cycle, lights on at 6:00 A.M.; temperature, 20–23 °C; relative humidity, 50–60%; aspen chip beddings) with food pellets (Harlan BV., Horst, Netherlands) and tap water available ad libitum. Young mice were assigned into treatment groups so that equal littermate distribution across the groups was ascertained. All procedures were approved by the Laboratory Animal Committee of the University of Helsinki and the Southern Finland Provincial Government, and carried out in accordance with the guidelines for experimental animal care.

2.2. Elevated plus-maze test for anxiety

To examine anxiety-related behaviors and sedation the mice were tested in an elevated plus-maze (EPM) as described earlier (Saarelainen et al., 2008). EPM was performed between 7:00–11:00 A.M. The plastic maze was elevated 54 cm from the floor level and it consisted of a central platform (5 cm × 5 cm), two open arms (5 cm × 40 cm with a 0.2 cm sides), and two closed arms (5 cm × 40 cm × 20 cm). The mice were injected with DZ i.p. at a dose of 0.5 mg/kg or 5 mg/kg. After injection the mice were returned to their home cages, and tested 20 min later on EPM. They were individually placed on the central platform facing the open arm and allowed free exploration of the maze for 5 min. Horizontal movements on EPM were recorded and analyzed by following the center of the animal's surface area from above using a video tracking system with EthoVision ColorPro 3.1 software (Noldus Information Technology, Wageningen, The Netherlands). An arm entry was recorded when the center of the mouse entered the arm at least 2 cm distal from the central platform.