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THE ROLE OF CHOLINERGIC BASAL FOREBRAIN NEURONS IN ADENOSINE-MEDIATED HOMEOSTATIC CONTROL OF SLEEP: LESSONS FROM 192 IGG–SAPORIN LESIONS

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Abstract—A topic of high current interest and controversy is the basis of the homeostatic sleep response, the increase in non-rapid-eye-movement (NREM) sleep and NREM-delta activity following sleep deprivation (SD). Adenosine, which accumulates in the cholinergic basal forebrain (BF) during SD, has been proposed as one of the important homeostatic sleep factors. It is suggested that sleep-inducing effects of adenosine are mediated by inhibiting the wake-active neurons of the BF, including cholinergic neurons. Here we examined the association between SD-induced adenosine release, the homeostatic sleep response and the survival of cholinergic neurons in the BF after injections of the immunotoxin 192 immunoglobulin G (IgG)–saporin (saporin) in rats. We correlated SD-induced adenosine level in the BF and the homeostatic sleep response with the cholinergic cell loss 2 weeks after local saporin injections into the BF, as well as 2 and 3 weeks after i.c.v. saporin injections.

Two weeks after local saporin injection there was an 88% cholinergic cell loss, coupled with nearly complete abolition of the SD-induced adenosine increase in the BF, the homeostatic sleep response, and the sleep-inducing effects of BF adenosine infusion.

Two weeks after i.c.v. saporin injection there was a 59% cholinergic cell loss, correlated with significant increase in SD-induced adenosine level in the BF and an intact sleep response. Three weeks after i.c.v. saporin injection there was an 87% cholinergic cell loss, nearly complete abolition of the SD-induced adenosine increase in the BF and the homeostatic response, implying that the time course of i.c.v. saporin lesions is a key variable in interpreting experimental results.

Taken together, these results strongly suggest that cholinergic neurons in the BF are important for the SD-induced increase in adenosine as well as for its sleep-inducing effects and play a major, although not exclusive, role in sleep homeostasis.

Key words: sleep deprivation, cholinergic lesion, delta activity, stereology, rat, immunotoxin.

The cholinergic basal forebrain (BF), adenosine and their relevance to sleep is a topic of high current interest. The BF contains cortically projecting wake-active neurons (Szymusiak et al., 2000; Semba, 2000; Jones, 2004) and, as referred in this paper, is composed of the horizontal diagonal band (HDB) substantia innominata (SI) and magnocellular preoptic nucleus (MCPO). Together with the medial septum (MS), vertical limb of the diagonal band (VDB), and the nucleus basalis magnocellularis (NBM), which also contain cholinergic neurons, these nuclei form a continuous cholinergic column in the BF.

In the BF, the somnogenic effects of the inhibitory neuromodulator, adenosine, have been suggested to be mediated via the A1 adenosine receptors (see Strecker et al., 2000; Basheer et al., 2004; McCarley 2007). *In vivo*, the BF extracellular adenosine was shown to increase gradually during sleep deprivation (SD), while the increase in homeostatic sleep response following SD (recovery sleep) was mimicked by increasing extracellular adenosine level with a transporter blocker, S-(p-nitrobenzyl)-6-thioinosine (NBTI) (Porkka-Heiskanen et al., 1997) and by adenosine infusion (Portas et al., 1997; Basheer et al., 1999). Adenosine, acting postsynaptically at the A1 receptor, inhibited BF cholinergic and some non-cholinergic neurons *in vitro* (Rainnie et al., 1994; Arrigoni et al., 2006) and inhibited BF wake-active neurons *in vivo* (Alam et al., 1999; Thakkar et al., 2003a), while antisense against the A1 receptor in the BF blocked the SD-induced increase in non-rapid-eye-movement (NREM) sleep and the increase in delta activity (Thakkar et al., 2003b). Taken together, these observations led to the hypothesis that BF adenosine accumulation during SD plays an important role in sleep homeostasis, promoting sleep by inhibiting BF wake-active neurons.

The BF contains several neurotransmitter phenotypes, including cortically projecting cholinergic, GABAergic and glutamatergic neurons (Manns et al., 2003; Steriade and McCarley, 2005). Cholinergic neurons were initially thought to be the major BF component promoting cortical activation/arousal since cortical acetylcholine release increased during cortical activation states of waking and rapid-eye movement (REM) sleep (Szerb, 1967; Marrosu et al., 1995) and blocking cholinergic receptors produced diminished cortical activation (Longo, 1966). These data led us to hypothesize that cholinergic neurons play an

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Abbreviations: AChE, acetylcholinesterase; ACSF, artificial cerebrospinal fluid; ANOVA, analysis of variance; BF, basal forebrain; ChAT, choline acetyltransferase; ChAT-ir, choline acetyltransferase immunoreactivity; EEG, electroencephalogram; EMG, electromyogram; GAD, glutamic acid decarboxylase; HDB, horizontal diagonal band; IgG, immunoglobulin G; MCPO, magnocellular preoptic nucleus; MS, medial septum; NBM, nucleus basalis magnocellularis; NBTI, S-(p-nitrobenzyl)-6-thioinosine; NF-κB, nuclear factor-κB; NREM, non-rapid-eye movement; PBS, phosphate buffer; REM, rapid-eye movement; RM-ANOVA, repeated measures analysis of variance; SD, sleep deprivation; SI, substantia innominata; VDB, vertical diagonal band.

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important but non-exclusive role in the BF adenosine actions, including sleep homeostasis.

However, the precise role of cholinergic neurons in adenosine-mediated homeostatic sleep control remained untested, and hence the interest in the use of the immunotoxin 192 immunoglobulin G (IgG)–saporin (saporin), a conjugate of a ribosomal inactivating enzyme, and the monoclonal antibody 192 IgG, which specifically binds to the p75 nerve growth factor-receptor located on BF cholinergic neurons and destroys them (Book et al., 1992; Heckers et al., 1994). Several studies which employed i.c.v. saporin injections have failed to detect stable significant changes in the sleep–wake cycle and the homeostatic sleep response when measured within 14–16 days post-injection (Bassant et al., 1995; Kapas et al., 1996; Gerashchenko et al., 2001; Blanco-Centurion et al., 2006). However, there are reports on the differential effect on the extent of cholinergic cell loss between rostral parts of BF (including MS and VDB) and the caudal nuclei of BF (including HDB, MCPO, SI and NBM) (Wrenn et al., 1999; Traissard et al., 2007; Moreau et al., 2008) after i.c.v. saporin injections. An almost complete loss of cholinergic cells located in the rostral areas was contrasted with only up to 60% of cholinergic cell loss in the caudal BF in these studies suggesting a slower time course for larger lesion development in caudal areas. This effect might be attributed to better diffusion of the toxin through the parenchyma to the rostral BF than to the caudal BF, which is more distant from the lateral ventricles (Moreau et al., 2008). Also bearing on measurements of the time course of effects, SD-induced adenosine levels in the HDB/SI/MCPO was not increased when measured 3 weeks after i.c.v. saporin injection, but these data were not correlated with measurements of recovery sleep at the same time point (Blanco-Centurion et al., 2006). In contrast to i.c.v. injections, which destroy cholinergic cells throughout the BF, the method of local saporin injections induces regionally precise destruction of cholinergic neurons (Pizzo et al., 1999). Studies using this method found minor changes in spontaneous sleep–waking cycles (Berntson et al., 2002; Kaur et al., 2008) but dramatic changes in the homeostatic sleep response (Kaur et al., 2008). However, to our knowledge, the effect of local injections on adenosine accumulation during SD as well as adenosine-induced sleep has not been studied.

The present study addressed the role of the BF cholinergic cells in adenosine-mediated homeostatic sleep control by using and comparing two different methods of saporin immunotoxin delivery, i.c.v. and local injection into HDB/SI/MCPO. We compared SD-induced adenosine increase in the BF, recovery sleep response after SD and adenosine-induced sleep in the same animals before and 2 weeks after local saporin injections. In order to examine whether the effects of i.c.v. saporin injections follow a slower time course, we measured SD-induced adenosine increase in the BF and recovery sleep response in the same animals both 2 and 3 weeks after i.c.v. saporin injections. We correlate our findings with the extent of cholinergic cell loss in HDB/SI/MCPO. Parts of these studies have appeared as abstracts (Kalinchuk et al., 2005, 2007).

EXPERIMENTAL PROCEDURES

This section first presents the experimental design and rationale, followed by description of methods and specific experimental details. All surgical and experimental protocols were approved by the Ethical Committee for Animal Experiments at the University of Helsinki and the provincial government of Uusimaa (Finland), were in accordance with the laws of Finland and the European Union and the Association for Assessment and Accreditation of Laboratory Animal Care and Use Committee at Boston VA Healthcare System, Harvard University and U.S. National Institutes of Health. Every effort was made to minimize animal suffering and to reduce the number of animals used.

Experimental design and rationale

Experiment 1: Investigation of the effects induced by local saporin administration on SD-induced adenosine accumulation and sleep homeostasis. The method of local, intraparenchymal injections of saporin allows performance of targeted lesion of cholinergic cells in the area of interest and avoidance of the extra-BF lesions (suprachiasmatic and cerebellar neurons) caused by i.c.v. saporin injection (Pizzo et al., 1999). Kaur et al. (2008) reported that bilateral local injections into the caudal part of BF (NBM/SI) resulted in attenuation of the homeostatic sleep response, but these authors did not measure changes in adenosine (Kaur et al., 2008). The present study used unilateral injections to determine if this minimal BF cholinergic lesion would be able to alter both adenosine accumulation during SD and the homeostatic sleep response. We reasoned that unilateral injections, if successful in altering sleep homeostasis, would be preferable to bilateral injection in that they would cause less nonspecific damage, and be in agreement with the general principle that the minimal lesion producing behavioral effects should be used. Although we were prepared to use bilateral injections if necessary, we thought there was a high likelihood that unilateral injections would be successful, since our previous studies had shown that unilateral pharmacological manipulations in the BF (HDB/SI/MCPO) such as adenosine infusion (Portas et al., 1997; Basheer et al., 1999), nucleoside transport blocker NBTI infusion (Porkka-Heiskanen et al., 1997), dinitrophenol application (Kalinchuk et al., 2003), and nitric oxide donor application (Kalinchuk et al., 2006a) were sufficient to cause marked changes in sleep. We studied the effects of local saporin injections on adenosine increase during 6 h SD by comparing adenosine values before and during SD, and simultaneously we studied the changes in recovery sleep following 6 h SD by measuring NREM sleep, NREM delta activity, and REM sleep. All measurements were performed in the same animals before and 2 weeks after saporin treatment (group L-SD-saporin, $N=6$). Another group of rats (group L-SD-saline, $N=5$) injected with saline served as treatment (injection) controls.

Experiment 2: Investigation of the effects induced by local saporin administrations on adenosine-induced sleep. Previously we have shown that unilateral administration of adenosine into BF induces sleep in the rat (Basheer et al., 1999). To determine the role of cholinergic neurons, if any, in mediating the somnogenic effects of adenosine, we infused adenosine into the BF by reverse microdialysis into the side ipsilateral to the saporin injection, in the same animals both before and 2 weeks after local saporin injec-