

Research Paper

Quinolate induces selective loss of melanin-concentrating hormone neurons, rather than orexin neurons, in the hypothalamus of mice and young rats

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Abbreviations:

GABA, γ -aminobutyric acid; MCH, melanin-concentrating hormone; NMDA, *N*-methyl-D-aspartate ; PBS, phosphate-buffered saline; QA, quinolinic acid; REM, rapid-eye-movement; ZT, Zeitgeber time.

Abstract

Orexins are neuropeptides produced in the lateral hypothalamus and implicated in regulation of sleep-wake cycle. Selective loss of orexin neurons is found in the brain of patients with narcolepsy, but the mechanisms of this pathological change are unclear. A previous study showed that excessive stimulation of *N*-methyl-D-aspartate (NMDA) receptors by quinolinic acid (QA) caused selective loss of orexin neurons in rat hypothalamic slice culture. Here we examined QA toxicity on orexin neurons and melanin-concentrating hormone (MCH) neurons in vivo. Contrary to the expectation, injection of QA (60 and 120 nmol) into the lateral hypothalamus of male C57BL/6 mice caused selective loss of MCH neurons rather than orexin neurons, and this toxicity of QA was attenuated by MK-801, an NMDA receptor antagonist. Selective loss of MCH neurons with preserved orexin neurons was observed even when GABA_A receptor antagonists such as bicuculline and picrotoxin were injected with QA. A significant decrease in the number of orexin neurons was induced when QA injection was performed in the dark phase of diurnal cycle, but the degree of the decrease was still lower than that in the number of MCH neurons. Finally, QA (60 nmol) induced selective loss of MCH neurons also in young rats at 3 - 4 weeks of age. These results do not support the hypothesis that acute excitotoxicity mediated by NMDA receptors is responsible for the pathogenesis of narcolepsy.

Keywords: excitotoxicity; hypocretin; narcolepsy; quinolinic acid

Orexins (also called as hypocretins) are neuropeptides that play an important role in regulation of sleep and wakefulness, neuroendocrine functions, food intake and energy homeostasis (Sakurai, 2007; Tsujino and Sakurai, 2009). Corresponding to these functions, neural activities of orexin neurons are elevated during active waking and motivated behaviors (Lee et al., 2005; Mileykovskiy et al., 2005). These neurons are localized in the lateral hypothalamic area, the brain region where melanin-concentrating hormone (MCH)-containing neurons are also distributed. Orexin neurons receive abundant projections from various regions of the brain (Sakurai, 2007). One of the inhibitory projections is from γ -aminobutyric acid (GABA)-containing neurons in the ventrolateral preoptic nucleus, which are sleep-active neurons and have a crucial role in initiation and maintenance of non-rapid-eye-movement (non-REM) sleep (Sakurai et al., 2005). Orexin neurons also seem to receive circadian influences indirectly from the suprachiasmatic nucleus, the central body clock (Tsujino and Sakurai, 2009). These interactions are important to regulate diurnal sleep–wake states.

Narcolepsy is a sleep disorder characterized by excessive daytime sleepiness or sleep attack and direct transition from wakefulness to REM sleep (Scammell, 2003). Narcolepsy affects about 1 in 2000 individuals worldwide and about 1 in 600 Japanese. The majority of narcolepsy patients suffer from cataplexy, i.e., loss of muscle tone triggered by emotion. Recent advances in basic and clinical research have identified selective loss of orexin neurons as a culprit of narcolepsy. That is, deletion of gene encoding preproorexin in mice results in appearance of symptoms reminiscent of human narcolepsy, such as fragmentation of awake/non-REM sleep episodes and reduced duration of wakefulness (Chemelli et al., 1999). Indeed, orexin concentrations in the cerebrospinal fluid samples from narcolepsy patients with

cataplexy are decreased to undetectable levels (Nishino et al., 2000, 2001). Moreover, post-mortem studies have shown that the number of orexin neurons in the lateral hypothalamus of narcolepsy patients is substantially lower than that in the same region of non-affected individuals, whereas the number of MCH neurons is unchanged (Peyron et al., 2000; Thannickal et al., 2000). So far, the mechanisms of selective loss of orexin neurons remain unclear.

A possible mechanism of neuron loss is *N*-methyl-D-aspartate (NMDA) receptor-mediated excitotoxicity. Quinolinic acid (QA), a tryptophan metabolite produced by the kynurenine pathway is among endogenous ligands at glutamate receptors. QA acts as an agonist at NMDA receptors and has been implicated in the pathogenesis of several neurodegenerative disorders such as Huntington disease (Stone, 2001). Notably, a previous study has shown that application of QA to hypothalamic slice cultures prepared from neonatal rat brain caused a preferential decrease of orexin neurons as compared with MCH neurons (Katsuki and Akaike, 2004). Accordingly, the present study was aimed to obtain further evidence that QA excitotoxicity could be involved in selective loss of orexin neurons in the hypothalamus. For this purpose, we examined the consequences of local microinjection of QA into the lateral hypothalamic area in vivo.

EXPERIMENTAL PROCEDURES

Drugs

QA and (+)-MK-801 maleate were obtained from Wako Chemicals (Osaka, Japan).

Picrotoxin, (–)-bicuculline methiodide and muscimol hydrobromide were obtained from

Sigma-Aldrich Chemicals (St. Louis, MO). QA free acid was dissolved at 400 mM in equimolar NaOH and diluted in phosphate-buffered saline (PBS) to give final concentrations. MK-801, picrotoxin, bicuculline and muscimol (the final concentration was 20, 4, 2 and 0.6 mM, respectively) were dissolved in PBS and mixed with QA solution at a final concentration of 120 or 240 mM. An inert tracer copper(II) phthalocyanine (1%, Sigma-Aldrich) was included in the solutions to be injected, for verification of the site of injection (Herrera et al., 2000).

Surgical procedures

All procedures were approved by our institutional animal experimentation committee, and animals were treated in accordance with the Guidelines of the United States National Institutes of Health regarding the care and use of animals for experimental procedures. All efforts were made to minimize the number of animals used and their suffering. Animals were housed in plastic cages with lights on from 8:00 to 20:00.

Intrahypothalamic administration to C57BL6/J mice was performed between 13:00 and 17:00, i.e., Zeitgeber time (ZT) 5 - ZT9. In the case of experiments on diurnal variations, the administration was performed between 10:00 and 11:00 in the light phase (ZT2 - ZT3) and between 22:00 and 23:00 in the dark phase (ZT14 - ZT15). Male C57BL6/J mice at ages between 8 and 10 weeks were anesthetized with pentobarbital (50 mg/kg i.p.). QA at 60 or 120 nmol in 0.5 μ l solution was injected through a 30-G injection cannula over a period of 2 min, into the lateral hypothalamus at the following stereotaxic coordinates: 2.0 mm posterior to bregma, 0.9 mm lateral to the midline and 5.0 mm below the dural surface, with the incisor bar at 3 mm above the interaural line. As a control, the same volume of PBS was injected

into the same stereotaxic coordinates in the contralateral hemisphere. In several sets of experiments, QA (60 or 120 nmol) was simultaneously injected with 2 nmol picrotoxin, 1 nmol bicuculline or 10 nmol MK-801, in a volume of 0.5 μ l.

Intrahypothalamic administration to Wistar rats was also performed between 13:00 and 17:00 (ZT5 - ZT9). Wistar rats at 3 - 4 weeks of age were anesthetized with pentobarbital, and QA solution (60 nmol/1 μ l) or PBS (1 μ l) was injected into the lateral hypothalamus (1 μ l over 4 min). QA was injected at the following coordinates: 2.5 mm posterior to bregma, 1.4 mm lateral to midline and 8.0 mm below the dural surface, with the incisor bar level with the interaural line.

After surgery, animals were recovered from anesthesia, and again housed in plastic cages with food and water available ad libitum.

Immunohistochemistry and cell counting

Four days after QA injection, mice and rats were anesthetized and perfused transcardially with PBS followed by 4% paraformaldehyde solution in phosphate buffer. Then the brain was isolated and post-fixed overnight with 4% paraformaldehyde, then immersed in 15% sucrose. After freezing, coronal sections of the hypothalamus at 30 μ m thickness were obtained every 120 μ m and mounted onto slides.

Brain sections were washed with PBS and then incubated in 10 mM sodium citrate buffer (pH 8.5) for 30 min at 78 °C for antigen retrieval. After cooling for 40 min, they were again washed with PBS, and blocked with 3% normal donkey serum in PBS containing 0.5% Triton X-100 for 1 h. Then, sections were incubated overnight in a primary antibody solution at 4 °C. Primary antibody solution consisted of goat polyclonal anti-orexin A (C-19) antibody

(1:300; sc-8070, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit MCH antiserum (1:20,000; H-070-47; Phoenix Pharmaceuticals, Belmont, CA, USA) in PBS containing 0.5% Triton X-100 and 3% normal donkey serum. After wash with PBS, sections were incubated in a secondary antibody solution for 2 h at room temperature. Secondary antibodies were Alexa Fluor 488-labeled donkey polyclonal anti-goat IgG (H+L) (1:200; A-11055, Molecular Probes, Eugene, OR, USA) and Alexa Fluor 594-labeled donkey polyclonal anti-rabbit IgG (H+L) (1:500; A-21207, Molecular Probes). Specimens were mounted onto slide glasses and coverslipped with 80% glycerol / 20% water, and fluorescence signals were observed with the use of an epifluorescence microscope. The number of immunopositive neurons in individual sections was obtained by surveying the entire area of each hemisphere of each section under the microscope at 200× magnification. Cells with discernible perinuclear immunoreactivity against respective neuropeptides were identified as orexin neurons and MCH neurons. The order of examinations on Alexa 488 and Alexa 594 dyes was random, and preliminary examinations using adjacent series of immunostained sections indicated that changing the order of examinations did not affect the outcome. The cell number in each hemisphere was presented as a summation of the values obtained from all sections encompassing the posterior hypothalamus. Abercrombie factor (Abercrombie, 1946), expressed as $[\text{section thickness}/(\text{section thickness} + \text{cell diameter})]$, was employed to correct the number of cells. Mean values of the diameter of cell bodies of orexin neurons and MCH neurons were 22.5 μm and 20.7 μm for mice, and were 21.1 μm and 19.6 μm for rats, respectively. We did not use anti-fade mounting media in the present study. Usage of Prolong Gold antifade reagent (Molecular Probes), one of the most suitable media for preserving photostability of both Alexa 488 and Alexa 594 dyes (Panchuk-Voloshina et al.,

1999), gave essentially the same results as those obtained with the usage of glycerol/water. Representative photographs of immunofluorescence presented in Fig. 1B and Fig. 4A were obtained by a laser-scanning confocal microscope system (Fluoview FV300; Olympus, Tokyo, Japan), and those in Fig. 1A and Fig. 5A were obtained by BIORIVO fluorescence microscope (BZ-9000; KEYENCE, Osaka, Japan).

Behavioral assessments

Mice were separated individually into plastic cages at least 3 days before surgery, and their body weight as well as the amount of food intake was measured once per day. Mice received bilateral injection of PBS/PBS, PBS/QA (120 nmol), and QA/QA (120 nmol for both sides) between 13:00 and 17:00 (ZT5 - ZT9), at the same stereotaxic coordinates as those described above (see Surgical Procedures). Four days after injection, locomotor activity of mice was examined in an open field of 500 mm × 500 mm. After habituation in the field for 30 min, locomotion during a 5-min period was measured using a video-tracking software (Limelight, Neuroscience, Tokyo, Japan). The test was performed between 18:00 and 20:00 (ZT10 - ZT12).

Statistical analysis

Data are expressed as means ± SEM. Student's *t*-test was used to compare the number of neurons between control (PBS-injected) and injured (QA-injected) hemisphere. For evaluation of the effect of drugs (MK-801, picrotoxin, bicuculline and muscimol) on QA toxicity, one-way analysis of variance followed by post-hoc Student-Newman-Keuls test was performed. Probability values less than 0.05 were considered statistically significant.

RESULTS

QA causes preferential loss of MCH neurons via NMDA receptor activation in adult mice

QA is an endogenous excitotoxin implicated in pathogenesis of several neurological disorders associated with neurodegeneration. Notably, application of this compound to hypothalamic slice cultures substantially decreased the number of viable orexin neurons while showing little effect on the number of MCH neurons (Katsuki and Akaike, 2004). Therefore, we performed unilateral microinjection of QA into the lateral hypothalamic area of adult mice, to clarify if QA produced selective cytotoxicity on orexin neurons. In this initial set of experiments, microinjection was performed between ZT5 and ZT9. The site of injection was located posterior to the region where orexin and MCH neurons were distributed, which enabled us to minimize influences of direct physical damage resulting from cannula insertion (Fig. 1A). Spreading of copper(II) phthalocyanine dye from the injection site was verified during preparation of frozen brain sections. We confirmed that regions containing orexin neurons were all covered by dye spreading. Contrary to our expectations, QA caused a preferential and dose-dependent decrease in the number of MCH neurons (Fig. 1B and C). Four days after injection of 60 nmol QA ($n = 5$), the number of orexin neurons in QA-injected side showed no significant difference from that in the control (vehicle-injected) side, whereas the number of MCH neurons in QA-injected side decreased by 41.4% as compared to that of the control side (left panel in Fig. 1C). The decrease in the number of MCH neurons was even more prominent when the dose of QA was increased to 120 nmol (by 60.5%, $n = 5$), but

again, the number of orexin neurons did not change significantly (right panel in Fig. 1C).

The decrease of MCH neurons by injection of 120 nmol QA was observed almost uniformly at every rostrocaudal level examined (Fig. 1D).

QA is well known as a weak NMDA receptor agonist (Stone, 2001) but might also exert several other biological actions. To examine whether NMDA receptor activation by QA was responsible for the decrease of MCH neurons, we used MK-801, a non-competitive NMDA receptor antagonist. Concomitant injection of 10 nmol MK801 significantly attenuated cytotoxicity of 120 nmol QA onto MCH neurons (Fig. 2; $n = 5$ for each group). Percentage reduction of MCH neurons as compared to the control side was 60.9% and 26.2% in the absence and the presence of MK-801, respectively.

GABA_A receptor blockade does not alter QA cytotoxicity

A major difference between in vitro and in vivo conditions is that distal projections making contact with orexin neurons are lost in slice cultures dissected from the hypothalamus. Lost projections include GABAergic inputs from the ventrolateral preoptic nucleus that provide inhibitory regulation on the activity level of orexin neurons (Sakurai et al., 2005). The level of neural activity may well influence the extent of NMDA receptor-mediated excitotoxicity. Indeed, exogenous application of GABA_A receptor agonists protects orexin neurons from QA cytotoxicity in hypothalamic slice culture (Katsuki and Akaike, 2005). We hypothesized that QA might be able to exert excitotoxicity on orexin neurons when GABAergic inhibitory inputs were shut off. To validate this hypothesis we used picrotoxin, a GABA_A receptor channel blocker (Fig. 3). Co-injection of 2 nmol picrotoxin did not exacerbate cytotoxicity of 60 nmol QA on either orexin neurons or MCH neurons ($n = 5$ for each group). Because

of the matter on solubility, we could not use picrotoxin at higher doses than 2 nmol.

Therefore, we also used bicuculline, a competitive GABA_A receptor antagonist. According to previous reports (Vezzani et al., 2000; Yamazaki et al., 2002), the dose of bicuculline was set to 1 nmol. However, bicuculline had no significant effect on cytotoxicity of 60 nmol QA (n = 5, Fig. 3).

Diurnal variation of QA cytotoxicity on orexin neurons

Neural activities of orexin neurons and MCH neurons exhibit diurnal variations. That is, orexin neurons show frequent spike activity during waking (i.e., during dark phase in the case of mice and rats) but are relatively silent during sleep (i.e., during light phase) (Lee et al., 2005; Mileykovskiy et al., 2005). In a reciprocal manner, MCH neurons are silent during waking and are active during sleep (Hassani et al., 2009). These variations of neural activities might affect the extent of excitotoxicity. Accordingly, we examined whether injection of QA during dark phase produced different consequences than that during light phase. When injection of QA at 60 or 120 nmol (n = 5 for each) was performed during early light phase (ZT2 – ZT3), QA induced a prominent and significant decrease in the number of MCH neurons. The number of orexin neurons was also slightly decreased by this treatment, but the effect did not reach statistical significance (Fig. 4B). These results were similar to those obtained with QA injection during later period of light phase (see Fig. 1). On the other hand, when QA was injected during early dark phase (ZT14 – ZT15), a significant decrease in the number of orexin neurons was observed (Fig. 4A and C). Percent reduction of the number of orexin neurons four days after injection of 60 and 120 nmol QA was 14.8% (n = 7) and 21.8% (n = 6), respectively. At the same time, however, the number of MCH neurons

also decreased. The decrease of MCH neurons (40.8% and 57.4% reduction by 60 and 120 nmol QA, respectively) was still more prominent than the decrease of orexin neurons. We also noted that the degree of the decrease of MCH neurons was not affected by the timing of QA injection.

Several lines of evidence indicate that orexin neurons located in different regions such as perifornical area and lateral hypothalamic area may play different physiological roles (Harris and Aston-Jones, 2006). We examined whether QA cytotoxicity on orexin neurons in the dark phase exhibited regional differences. Similar to the study by Murphy et al. (2003), hypothalamic areas were divided into three contiguous rectangles ($500\ \mu\text{m} \times 800\ \mu\text{m}$) for cell counting that roughly corresponded to dorsomedial hypothalamus, perifornical area, and lateral hypothalamic area, respectively (Fig. 5A). We found that the number of orexin neurons in the lateral hypothalamic area was substantially decreased by injection of 120 nmol QA during early dark phase (ZT14 - ZT15), whereas the number of orexin neurons in the other areas was not affected significantly ($n = 6$, Fig. 5B). We also confirmed that 120 nmol QA did not cause significant cytotoxicity on orexin neurons when a GABA_A receptor agonist muscimol (0.3 nmol) was simultaneously injected ($n = 6$ for QA alone, and $n = 5$ for QA plus muscimol; Fig. 5C). In contrast, QA cytotoxicity on MCH neurons was not attenuated by muscimol.

QA causes preferential loss of MCH neurons in young rats

Next we examined whether the different properties of QA cytotoxicity observed in vivo and in vitro were based on differences of species and age of animals. The previous in vitro study used hypothalamic slice cultures that were prepared from Wistar rats at 8 to 9 days old and

were cultured for 17 - 20 days until drug treatment (Katsuki and Akaike, 2004). To match the species an age of animals with those of the previous study, we used Wistar rats at 3 - 4 weeks of age ($n = 5$), and injected 60 nmol QA into the lateral hypothalamic area. We found that, similar to the case with mice, QA caused a significant decrease in the number of MCH neurons (37.7% reduction at four days after injection) while showing no significant effect on the number of orexin neurons (Fig. 6).

Behavioral parameters after QA-induced lesion

Mice lacking MCH (Shimada et al., 1998) and mice with targeted ablation of MCH neurons (Alon and Friedman, 2006) have been reported to exhibit hypophagia and reduced body weight. We examined if QA-induced lesion in the hypothalamus resulted in similar phenotypes to those observed in MCH-deficient mice. In this set of experiments, sham-operated mice received bilateral injection of PBS ($n = 5$), whereas other mice received injection of 120 nmol QA in one hemisphere ($n = 5$). These two groups of mice showed no significant differences each other, with respect to body weight and food intake during the course of two weeks after surgery (Fig. 7A). In addition, they showed no difference in locomotor activity assessed four days after surgery (Fig. 7B). We could not evaluate properly the consequences of lesion of both hemispheres of the hypothalamus, because bilateral injection of 120 nmol QA resulted in high mortality of mice (five out of ten mice died within four days after surgery), and surviving mice showed a marked reduction in locomotor activity (data not shown).

DISCUSSION

In the brain of narcolepsy patients, orexin neurons in the hypothalamus are substantially decreased, whereas MCH neurons in the same area are preserved (Peyron et al., 2000; Thannickal et al., 2000). The mechanisms of selective loss of orexin neurons remain unsolved. Indeed, very few studies addressed this issue directly. Gerashchenko and Shiromani (2004) reported that chronic infusion of lipopolysaccharide into the lateral hypothalamus of adult rats resulted in a significant decrease in the number of orexin neurons, and proposed that inflammation may play a role in the loss of orexin neurons in narcolepsy. In their study, however, the number of MCH neurons also decreased to a similar extent, which means that selective loss of orexin neurons is not produced by this treatment. On the other hand, a prevailing view on the pathogenesis of narcolepsy is possible involvement of autoimmune responses against orexin neurons: several lines of evidence support this proposal (Overeem et al., 2008; Hallmayer et al., 2009; Cvetkovic-Lopes et al., 2010), although direct evidence is still lacking.

The focus of the present study was to determine whether excitotoxicity could produce selective loss of orexin neurons *in vivo*. Excitotoxicity has been implicated in degeneration of specific subsets of neurons in various neurodegenerative disorders (Doble, 1999) and also in neurodegeneration associated with autoimmune processes (Smith et al., 2000; DeGiorgio et al., 2001). The strong evidence for possible involvement of excitotoxicity in selective loss of orexin neurons comes from a previous study by one of us (Katsuki and Akaike, 2004), where QA as well as NMDA caused preferential loss of orexin neurons as compared to MCH neurons in rat hypothalamic slice culture. Unexpectedly, the present study revealed that QA cytotoxicity *in vivo* exhibited totally different properties than that *in vitro*, in terms of neuron

selectivity. Under all conditions tested, MCH neurons decreased to a greater extent than orexin neurons. QA cytotoxicity on MCH neurons was attenuated by MK-801, indicating that NMDA receptor activation was responsible for the decrease in orexin neurons.

Because we found preferential loss of MCH neurons by QA in the initial set of experiments, we explored other experimental conditions that might affect excitotoxic consequences of QA injection toward loss of orexin neurons. A notable difference of orexin neurons in hypothalamic slice cultures from those *in vivo* is that they have lost neural connections involving brain regions outside the posterior hypothalamus. Particularly, GABAergic projection neurons originating from the ventrolateral preoptic nucleus may exert profound influences on neural activity of orexin neurons (Sakurai et al., 2005). Therefore, we examined the effects of GABA_A receptor antagonists on QA cytotoxicity. As a result, preferential loss of MCH neurons by QA was observed even when picrotoxin or bicuculline was co-administered with QA. Hence, GABAergic inhibitory regulation onto orexin neurons may not be a major factor contributing to the resistance of orexin neurons against excitotoxicity *in vivo*.

The above result does not exclude potential influences of neural connections on excitotoxic consequences in orexin neurons. Orexin neurons receive several inhibitory inputs other than GABAergic projections, including those from noradrenaline-containing neurons in the locus coeruleus and serotonin-containing neurons in the raphe nuclei (Sakurai, 2007; Tsujino and Sakurai, 2009). In addition, the suprachiasmatic nucleus sends inputs to orexin neurons via dorsomedial hypothalamus (Tsujino and Sakurai, 2009). Depending on these connections, neural activities of orexin neurons exhibit diurnal variations (Lee et al., 2005; Mileykovskiy et al., 2005). Because NMDA receptor channels open only when

voltage-dependent Mg^{2+} block is relieved, NMDA receptor-mediated excitotoxicity may be readily induced when neuronal excitability is elevated. We found here that QA could cause a significant loss of orexin neurons when administered during early dark phase, which was abrogated by concomitant application of muscimol, a $GABA_A$ receptor agonist. This is consistent with the fact that orexin neurons in mice and rats are active during dark phase. Interestingly, however, orexin neurons that were most vulnerable to QA cytotoxicity during dark phase were those located in the lateral hypothalamic area. These observations are not simply related to the proposal by Harris and Aston-Jones (2003) that orexin neurons in the perifornical and dorsomedial hypothalamus regulate arousal, whereas those in the lateral hypothalamus regulate reward processing.

Heightened vulnerability of MCH neurons to QA cytotoxicity as compared to orexin neurons was consistently observed, even when we used young rats at 3 - 4 weeks of age. Developmental processes in the hypothalamus have almost ended by this age, although sexual maturation may continue (Shimogori et al., 2010). We selected this age of rats because the previous study in vitro (Katsuki and Akaike, 2004) relied on results obtained from hypothalamic slices of 8 - 9 day-old rats that were cultured for 17 - 20 days. The present results suggest that differences in age and animal species do not explain discrepancy between the results in vivo and those in vitro.

The reasons why MCH neurons in vivo are vulnerable to QA cytotoxicity are ~~also~~ unclear. An electrophysiological study on mouse hypothalamic slices has demonstrated that MCH neurons possess NMDA receptors and respond to NMDA with depolarization (Huang and van den Pol, 2007). In hypothalamic slice culture, these neurons are resistant to cytotoxicity of NMDA and QA as compared to orexin neurons (Katsuki and Akaike, 2004), which might be

related to the fact that MCH neurons in vitro are electrically silent (Eggermann et al., 2003). However, this scenario may not apply to QA cytotoxicity on MCH neurons in vivo, because the degree of QA cytotoxicity showed no apparent diurnal variations in spite of reported variations in neural activity of MCH neurons during sleep-wake cycle (Hassani et al., 2009). Reasons for ineffectiveness of muscimol in rescuing MCH neurons from QA cytotoxicity are also unclear, although there are several reported cases where GABA_A receptor activation did not affect or even exacerbated excitotoxic injury (Erdo et al., 1991; Yoshikawa et al., 1998). Although further work is required, vulnerability of MCH neurons to excitotoxicity might be taken into account for the pathology of several neurological disorders associated with dysregulation of food intake and sleep. MCH has been suggested to play an important role in regulation of paradoxical sleep (Peyron et al., 2009), and MCH-deficient mice sleep about 90 min less than wild-type mice daily (Willie et al., 2008). With respect to food intake, MCH neurons interact with other hypothalamic neurons critically involved in regulation of feeding behavior and energy homeostasis (Griffond and Risold, 2009). Mice lacking MCH (Shimada et al., 1998) and mice with targeted ablation of MCH neurons (Alon and Friedman, 2006) exhibit mild hypophagia and reduced body weight. In the present study, however, we did not find any significant differences between sham-operated mice and QA-treated mice concerning food intake and body weight. This may be largely attributable to the fact that our experimental model was based on hemi-lesion, leaving MCH neurons in one hemisphere intact.

In conclusion, the present study proposes that acute excitotoxic injury may not be primarily responsible for the pathogenesis of narcolepsy. The clearly different results in whole animals from those in organotypic slice cultures casts a caution, with respect to

neurodegenerative processes of hypothalamic orexin neurons, to extrapolate in vitro findings to in vivo situations.

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REFERENCES

- Abercrombie M (1946) Estimation of nuclear population from microtome sections. *Anat Rec* 94:239-247.
- Alon T, Friedman JM. (2006) Late-onset leanness in mice with targeted ablation of melanin concentrating hormone neurons. *J Neurosci* 26:389-397.
- Chemelli RM, Willie JT, Sinton CM, Elmquist JK, Scammell T, Lee C, Richardson JA, Williams SC, Xiong Y, Kisanuki Y, Fitch TE, Nakazato M, Hammer RE, Saper CB, Yanagisawa M (1999) Narcolepsy in orexin knockout mice: molecular genetics of sleep regulation. *Cell* 98:437-451.
- Cvetkovic-Lopes V, Bayer L, Dorsaz S, Maret S, Pradervand S, Dauvilliers Y, Lecendreux M, Lammers GJ, Donjacour CE, Du Pasquier RA, Pfister C, Petit B, Hor H, Mühlethaler M, Tafti M (2010) Elevated Tribbles homolog 2-specific antibody levels in narcolepsy patients. *J Clin Invest* 120:713-719.
- DeGiorgio LA, Konstantinov KN, Lee SC, Hardin JA, Volpe BT, Diamond B (2001) A subset of lupus anti-DNA antibodies cross-reacts with the NR2 glutamate receptor in systemic lupus erythematosus. *Nat Med* 7:1189-1193.
- Doble A (1999) The role of excitotoxicity in neurodegenerative disease: implications for therapy. *Pharmacol Ther* 81:163-221.
- Eggermann E, Bayer L, Serafin M, Saint-Mleux B, Bernheim L, Machard D, Jones BE, Mühlethaler M (2003) The wake-promoting hypocretin-orexin neurons are in an intrinsic state of membrane depolarization. *J Neurosci* 23:1557-1562.
- Erdo S, Michler A, Wolff JR (1991) GABA accelerates excitotoxic cell death in cortical cultures: protection by blockers of GABA-gated chloride channels. *Brain Res*

542:254-258.

Gerashchenko D, Shiromani PJ (2004) Effects of inflammation produced by chronic lipopolysaccharide administration on the survival of hypocretin neurons and sleep. *Brain Res* 1019:162-169.

Griffond B, Risold PY (2009) MCH and feeding behavior-interaction with peptidic network. *Peptides* 30:2045-2051.

Hallmayer J, Faraco J, Lin L, Hesselson S, Winkelmann J, Kawashima M, Mayer G, Plazzi G, Nevsimalova S, Bourgin P, Hong SC, Honda Y, Honda M, Högl B, Longstreth WT Jr, Montplaisir J, Kemlink D, Einen M, Chen J, Musone SL, Akana M, Miyagawa T, Duan J, Desautels A, Erhardt C, Hesla PE, Poli F, Frauscher B, Jeong JH, Lee SP, Ton TG, Kvale M, Kolesar L, Dobrovolná M, Nepom GT, Salomon D, Wichmann HE, Rouleau GA, Gieger C, Levinson DF, Gejman PV, Meitinger T, Young T, Peppard P, Tokunaga K, Kwok PY, Risch N, Mignot E (2009) Narcolepsy is strongly associated with the T-cell receptor alpha locus. *Nat Genet* 41:708-711.

Harris GC, Aston-Jones G (2006) Arousal and reward: a dichotomy in orexin function. *Trends Neurosci.* 29:571-577.

Hassani OK, Lee MG, Jones BE (2009) Melanin-concentrating hormone neurons discharge in a reciprocal manner to orexin neurons across the sleep-wake cycle. *Proc Natl Acad Sci USA* 106:2418-2422.

Herrera AJ, Castaño A, Venero JL, Cano J, Machado A (2000) The single intranigral injection of LPS as a new model for studying the selective effects of inflammatory reactions on dopaminergic system. *Neurobiol Dis* 7:429-447.

Huang H, van den Pol AN (2007) Rapid direct excitation and long-lasting enhancement of

- NMDA response by group I metabotropic glutamate receptor activation of hypothalamic melanin-concentrating hormone neurons. *J Neurosci* 27:11560-11572.
- Katsuki H, Akaike A (2004) Excitotoxic degeneration of hypothalamic orexin neurons in slice culture. *Neurobiol Dis* 15:61-69.
- Katsuki H, Akaike A (2005) Quinolinic acid toxicity on orexin neurons blocked by gamma aminobutyric acid type A receptor stimulation. *Neuroreport* 16:1157-1161.
- Lee MG, Hassani OK, Jones BE (2005) Discharge of identified orexin/hypocretin neurons across the sleep-waking cycle. *J Neurosci* 25:6716-6720.
- Mileykovskiy BY, Kiyashchenko LI, Siegel JM (2005) Behavioral correlates of activity in identified hypocretin/orexin neurons. *Neuron* 46:787-798.
- Murphy JA, Deurveilher S, Semba K (2003) Stimulant doses of caffeine induce c-FOS activation in orexin/hypocretin-containing neurons in rat. *Neuroscience* 121:269-275.
- Nishino S, Ripley B, Overeem S, Lammers GJ, Mignot E (2000) Hypocretin (orexin) deficiency in human narcolepsy. *Lancet* 355:39-40.
- Nishino S, Ripley B, Overeem S, Nevsimalova S, Lammers GJ, Vankova J, Okun M, Rogers W, Brooks S, Mignot E (2001) Low cerebrospinal fluid hypocretin (orexin) and energy homeostasis in human narcolepsy. *Ann Neurol* 50:381-388.
- Overeem S, Black JL 3rd, Lammers GJ (2008) Narcolepsy: immunological aspects. *Sleep Med Rev* 12:95-107.
- Panchuk-Voloshina N, Haugland RP, Bishop-Stewart J, Bhalgat MK, Millard PJ, Mao F, Leung WY, Haugland RP (1999) Alexa dyes, a series of new fluorescent dyes that yield exceptionally bright, photostable conjugates. *J Histochem Cytochem* 47:1179-1188.

- Peyron C, Faraco J, Rogers W, Ripley B, Overeem S, Charnay Y, Nevsimalova S, Aldrich M, Reynolds D, Albin R, Li R, Hungs M, Pedrazzoli M, Padigaru M, Kucherlapati M, Fan J, Maki R, Lammers GJ, Bouras C, Kucherlapati R, Nishino S, Mignot E (2000) A mutation in a case of early onset narcolepsy and a generalized absence of hypocretin peptides in human narcoleptic brains. *Nat Med* 6:991-997.
- Peyron C, Sapin E, Leger L, Luppi PH, Fort P (2009) Role of the melanin-concentrating hormone neuropeptide in sleep regulation. *Peptides* 30:2052-2059.
- Sakurai T (2007) The neural circuit of orexin (hypocretin): maintaining sleep and wakefulness. *Nat Rev Neurosci* 8:171-181.
- Sakurai T, Nagata R, Yamanaka A, Kawamura H, Tsujino N, Muraki Y, Kageyama H, Kunita S, Takahashi S, Goto K, Koyama Y, Shioda S, Yanagisawa M (2005) Input of orexin/hypocretin neurons revealed by a genetically encoded tracer in mice. *Neuron* 46:297-308.
- Scammell TE (2003) The neurobiology, diagnosis, and treatment of narcolepsy. *Ann Neurol* 53:154-166.
- Shimada M, Tritos NA, Lowell BB, Flier JS, Maratos-Flier E (1998) Mice lacking melanin-concentrating hormone are hypophagic and lean. *Nature* 396:670-674.
- Shimogori T, Lee DA, Miranda-Angulo A, Yang Y, Wang H, Jiang L, Yoshida AC, Kataoka A, Mashiko H, Avetisyan M, Qi L, Qian J, Blackshaw S (2010) A genomic atlas of mouse hypothalamic development. *Nat Neurosci* 13:767-775.
- Smith T, Groom A, Zhu B, Turski L (2000) Autoimmune encephalomyelitis ameliorated by AMPA antagonists. *Nat Med* 6:62-66.
- Stone TW (2001) Kynurenines in the CNS: from endogenous obscurity to therapeutic

- importance. *Prog Neurobiol* 64:185-218.
- Sutcliffe JG, de Lecea L (2002) The hypocretins: setting the arousal threshold. *Nat Rev Neurosci* 3:339-349.
- Thannickal TC, Moore RY, Nienhuis R, Ramanathan L, Gulyani S, Aldrich M, Cornford M, Siegel JM (2000) Reduced number of hypocretin neurons in human narcolepsy. *Neuron* 27:469-474.
- Tsujino N, Sakurai T (2009) Orexin/hypocretin: a neuropeptide at the interface of sleep, energy homeostasis, and reward system. *Pharmacol Rev* 61:162-176.
- Vezzani A, Moneta D, Conti M, Richichi C, Ravizza T, De Luigi A, De Simoni MG, Sperk G, Andell-Jonsson S, Lundkvist J, Iverfeldt K, Bartfai T (2000) Powerful anticonvulsant action of IL-1 receptor antagonist on intracerebral injection and astrocytic overexpression in mice. *Proc Natl Acad Sci USA* 97:11534-11539.
- Willie JT, Sinton CM, Maratos-Flier E, Yanagisawa M (2008) Abnormal response of melanin-concentrating hormone deficient mice to fasting: hyperactivity and rapid eye movement sleep suppression. *Neuroscience* 156:819-829.
- Yamazaki S, Mochizuki Y, Terai T, Sugimoto M, Uchida I, Matsuoka N, Mutoh S (2002) Intracerebroventricular injection of the antibiotic cefoselis produces convulsion in mice via inhibition of GABA receptors. *Pharmacol Biochem Behav* 74:53-59.
- Yoshikawa M, Goto S, Okamura A, Hamasaki T, Ushio Y (1998) Neurotoxicity evoked by *N*-methyl-D-aspartate in the organotypic static slice cultures of rat cerebral cortices: effect of GABA_A receptor activation. *Acta Neuropathol* 95:592-596.

Figure Legends

Fig. 1. QA causes preferential loss of MCH neurons in mice. (A) Shown are a representative coronal brain section containing the injection site (2.0 mm posterior to bregma; left panel) and sections obtained from the coordinates where orexin and MCH neurons are distributed (1.2 mm posterior to bregma; middle and right panels). Sections in left and middle panels were stained with cresyl violet (Nissl staining). The section in the right panel shows orexin immunofluorescence. Arrows indicate the site of injection. Note that the area containing orexin neurons did not undergo physical or necrotic damage by injection cannula. (B) Representative photomicrographs showing orexin (in green) and MCH (in magenta) immunoreactivities in the lateral hypothalamic area. Immunoreactivities were examined four days after injection of vehicle in the control side and 60 or 120 nmol QA in the contralateral side. Injection was performed between ZT5 and ZT9. (C) Effect of QA injected at 60 nmol (left panel, $n = 5$) and 120 nmol (right panel, $n = 5$) on the number of orexin neurons and MCH neurons. *** $P < 0.001$ vs. control. (D) An example of distribution of orexin- (left panel) and MCH- (right panel) immunoreactive neurons as expressed by the number of immunopositive cells in each section. Examined sections 1 -17 correspond to coordinates 2.3 - 0.2 mm posterior to bregma. The coordinate corresponding to the injection site (2.0 mm posterior to bregma) is indicated by an arrow.

Fig. 2. Effect of MK-801 on QA cytotoxicity. Orexin- and MCH-immunoreactivities were examined four days after injection of 120 nmol QA ($n = 5$) or 120 nmol QA plus 10 nmol MK-801 ($n = 5$). Injection was performed between ZT5 and ZT9. *** $P < 0.001$ vs.

control; ### $P < 0.001$ vs. QA alone (ANOVA results: for orexin neurons, $F_{2,17} = 2.864$, $P = 0.0847$; for MCH neurons, $F_{2,17} = 38.72$, $P < 0.0001$).

Fig. 3. GABA_A receptor blockade does not alter QA-induced selective loss of MCH neurons. Orexin- and MCH-immunoreactivities were examined four days after injection of 60 nmol QA (n = 5), 60 nmol QA plus 2 nmol picrotoxin (PTX, n = 5) or 60 nmol QA plus 1 nmol bicuculline (Bic., n = 5). Injection was performed between ZT5 and ZT9. *** $P < 0.001$ vs. control (ANOVA results: for orexin neurons, $F_{3,26} = 1.952$, $P = 0.146$; for MCH neurons, $F_{3,26} = 27.32$, $P < 0.0001$).

Fig. 4. Diurnal variations of QA cytotoxicity. (A) Representative photomicrographs showing orexin (in green) and MCH (in magenta) immunoreactivities in the lateral hypothalamic area. Immunoreactivities were examined four days after injection of vehicle in the control side and 120 nmol QA in the contralateral side. Injection was performed during early dark phase, i.e., between ZT14 and ZT15. (B) Effect of QA injected at 60 nmol (left panel, n = 5) and 120 nmol (right panel, n = 5) during early light phase (ZT2 - ZT3) on the number of orexin- and MCH-immunoreactive neurons. *** $P < 0.001$ vs. control. (C) Effect of QA injected at 60 nmol (left panel, n = 7) and 120 nmol (right panel, n = 6) during early dark phase (ZT14 - ZT15) on the number of orexin- and MCH-immunoreactive neurons. * $P < 0.05$, *** $P < 0.001$ vs. control.

Fig. 5. QA cytotoxicity on orexin neurons during early dark phase. (A) A representative section showing the location of rectangles for cell counting (500 $\mu\text{m} \times 800 \mu\text{m}$).

Abbreviations: DMH, dorsomedial hypothalamus; PeF, perifornical area; LH, lateral hypothalamic area. (B) The number of orexin neurons within three rectangles was examined four days after injection of 120 nmol QA during early dark phase (ZT14 - ZT15). $n = 6$. *** $P < 0.001$ vs. control. (C) Effect of muscimol on QA cytotoxicity during early dark phase. Orexin- and MCH-immunoreactivities were examined four days after injection of 120 nmol QA ($n = 6$), or 120 nmol QA plus 0.3 nmol muscimol ($n = 6$). Injection was performed between ZT14 and ZT15. * $P < 0.05$, *** $P < 0.001$ vs. control (ANOVA results: for orexin neurons, $F_{2,19} = 4.067$, $P = 0.034$; for MCH neurons, $F_{2,19} = 134.82$, $P < 0.0001$).

Fig. 6. QA cytotoxicity in young rats. The number of orexin- and MCH-immunoreactive neurons was examined four days after injection of vehicle in the control side and 60 nmol QA in the contralateral side of the lateral hypothalamic area of rats at 3 - 4 weeks of age. Injection was performed between ZT5 and ZT9. $n = 5$. ** $P < 0.01$ vs. control.

Fig. 7. Behavioral parameters after QA-induced lesion. Sham-operated control mice received bilateral injection of PBS into the hypothalamus, whereas QA-treated mice received injection of 120 nmol QA in one hemisphere and PBS in the other. Injection was performed between ZT5 and ZT9. $n = 5$ for each group. (A) Body weight changes (left panel) and amount of daily food intake (right panel) after surgery at day 0. (B) Locomotor activity in an open field was examined at four days after surgery. Values in the graph represent total distance traveled during a 5-min observation period. Traces shown on the right are representative tracks of locomotion.