



Neuropharmacology and analgesia

Barbiturates enhance itch-associated scratching in atopic dermatitis mice: A possible clue to understanding nocturnal pruritus in atopic dermatitis



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ABSTRACT

In chronic pruritic diseases such as atopic dermatitis, pruritus is exacerbated during nocturnal sleep; however, the underlying mechanism remains unclear. We previously demonstrated that acute administration of the sedative-hypnotics ethanol markedly enhanced itch-associated spontaneous scratching in a diet-induced mouse model of atopic dermatitis. In the present study, to expand our previous finding and provide a general mechanism for the central modulation of chronic itch, we examined whether other hypnotic drugs, such as barbiturates and benzodiazepines, also enhance scratching, and further investigated the underlying mechanism. Barbiturates markedly enhanced spontaneous scratching in the atopic dermatitis model but not controls. However, unexpectedly, benzodiazepines only slightly increased scratching, and the selective γ -aminobutyric acid type A (GABA_A) receptor agonist, muscimol, had no effect. Local injection studies have demonstrated that barbiturates act at the supraspinal level to enhance scratching. Barbiturate-induced scratching was inhibited not only by GABA_A receptor antagonists but also by an L-type voltage-dependent calcium channel (L-VDCC) agonist and an α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptor agonist. An intracisternally injected AMPA receptor antagonist alone or in combination with an L-VDCC antagonist sufficiently enhanced scratching. Barbiturate-induced scratching enhancement was observed in another atopic dermatitis model, NC/Nga, but not in histamine-induced acute itch model in normal healthy mice. Overall, our results suggest that a synergistic effect among AMPA receptor inhibition, GABA_A receptor activation, and L-VDCC inhibition in the brain mediates barbiturate-induced scratching in atopic dermatitis mice. This observation may provide a novel clue to understanding a supraspinal itch mechanism in chronic diseases such as atopic dermatitis.

1. Introduction

Itch (or pruritus) is an unpleasant sensation that evokes a desire to scratch. Itch signals are transmitted from the periphery to the central nervous system (CNS), likely by dedicated neurons distinct from those that transmit pain (Carstens and Akiyama, 2016). Although remarkable progress has been made over the last decade with regard to the understanding of neuronal molecular mechanisms of itch in the periphery and spinal cord (Barry et al., 2018; Dong and Dong, 2018), the molecules and mechanisms that mediate and regulate itch in the supraspinal CNS remain poorly understood.

Atopic dermatitis is the most common chronic inflammatory skin disease. Pruritus is the major symptom of this disease; however, its

management remains challenging (Elmariah, 2017). In atopic dermatitis, pruritus is exacerbated during nocturnal sleep, resulting in sleep disturbance, thereby significantly reducing the patient's quality of life (Ebata et al., 1999; Yosipovitch et al., 2002). Moreover, chronic scratching worsens skin conditions and leads to a vicious itch-scratch cycle (Wahlgren, 1999). Thus, control of nocturnal pruritus is of vital importance in the treatment of atopic dermatitis. Although some mechanisms have been proposed to account for nocturnal pruritus (Lavery et al., 2016; Patel et al., 2007), the exact molecular mechanism is not fully understood.

We previously reported a unique, diet-induced mouse model of atopic dermatitis. HR-1 hairless mice fed a special diet (named HR-AD) exhibited atopic dermatitis-like skin inflammation accompanied by

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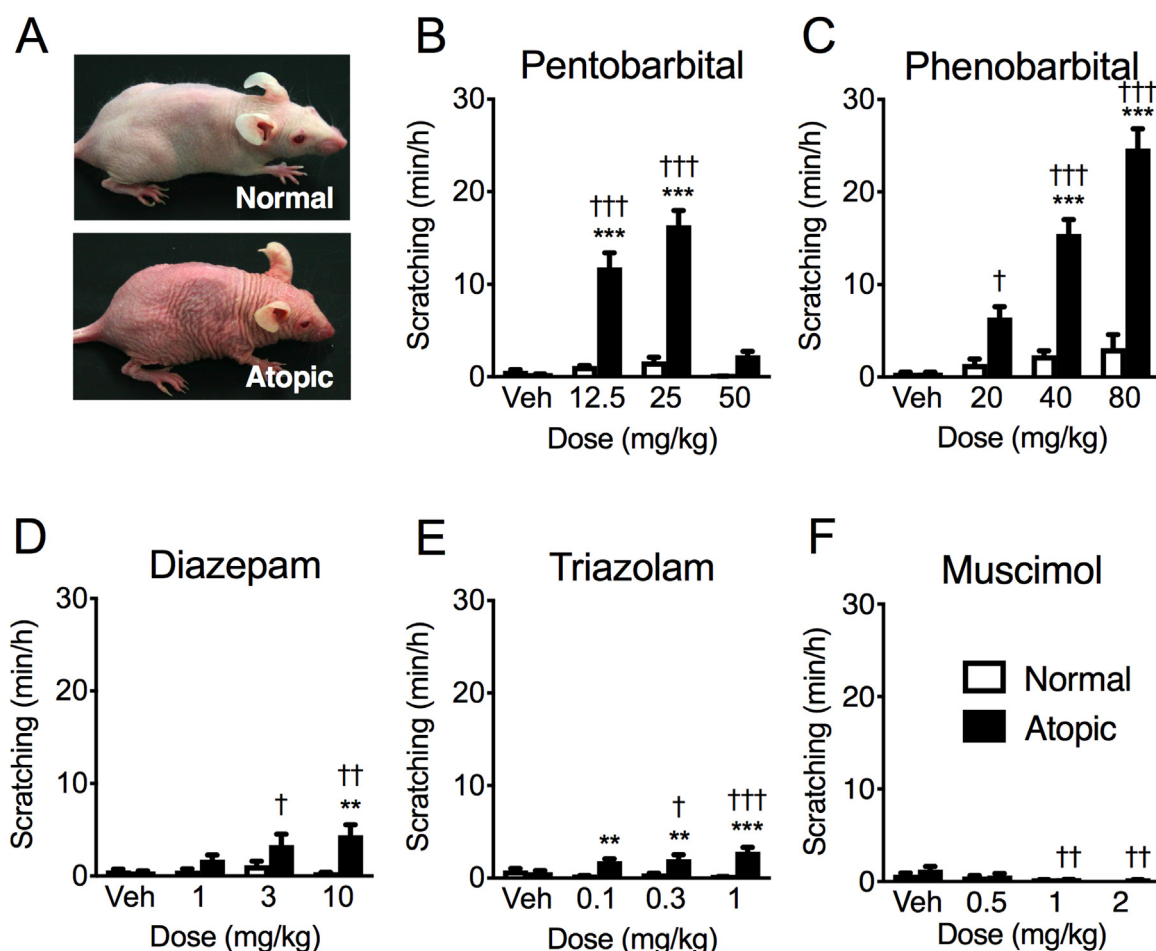


Fig. 1. Effects of barbiturates, benzodiazepines, and muscimol on scratching behavior in normal and atopic mice. (A) Mouse fed a normal diet (upper, designated as Normal) or the special diet (bottom, designated as Atopic) for 11 weeks. (B–F) Vehicle [saline (B, C, and F) and purified water containing 0.3% Tween 80 (D and E)], pentobarbital (12.5, 25, and 50 mg/kg, i.p.) (B), phenobarbital (20, 40, and 80 mg/kg, i.p.) (C), diazepam (1, 3, and 10 mg/kg, p.o.) (D), triazolam (0.1, 0.3, and 1 mg/kg, p.o.) (E), or muscimol (0.5, 1, and 2 mg/kg, i.p.) (F) was administered to normal and atopic mice. Immediately after administration, scratching behavior was measured for 1 h. Each column represents the mean $\pm$ S.E.M. of eight to ten (B), six or seven (C), six or seven (D), eight to 11 (E), or four to seven (F) animals. ** $P < 0.01$ and *** $P < 0.001$, Normal vs. Atopic, and † $P < 0.05$, †† $P < 0.01$ and ††† $P < 0.001$, vs. Vehicle (Veh).

spontaneous itch-associated scratching (Fujii et al., 2005, 2006, 2015). Since intake of alcohol was known to be one of triggers for itch in atopic dermatitis (Ständer and Steinhoff, 2002), we previously examined the effect of ethanol on scratching and found that oral administration of a high dose of ethanol (2.4 g/kg) markedly enhanced scratching in atopic but not normal mice (Fujii et al., 2009). Pharmacological analysis revealed that both γ -aminobutyric acid type A (GABA_A) receptor activation and *N*-methyl-D-aspartate glutamate receptor inhibition in the CNS contributed to the ethanol-induced scratching (Fujii et al., 2009). As ethanol is a CNS depressant and can induce a hypnotic effect, we assumed that enhancement of scratching by certain CNS depressants in atopic mice mimics the nocturnal pruritus frequently observed in the patients.

In the present study, to expand our previous finding and provide a general mechanism for CNS modulation of chronic itch, we addressed the following questions: (1) Do other hypnotic drugs, such as barbiturates and benzodiazepines, also enhance scratching in atopic dermatitis model mice? (2) Where does the drug act within the nervous systems to enhance scratching? (3) Is such enhancement of scratching mediated exclusively by GABA_A receptor activation? If not, which neurotransmitters and receptors are involved? (4) Is such enhancement of scratching observed not only in chronic disease conditions but also universally in acute itch in healthy conditions? The answers to these questions could further advance our understanding of the mechanisms

underlying chronic pruritus.

2. Materials and methods

2.1. Animals

Four-week-old, female hairless mice (Hos: HR-1) were purchased from Hoshino Laboratory Animals (Saitama, Japan). Additionally, 10-week-old, male NC/Nga mice that were healthy or had developed dermatitis were purchased from Japan SLC (Shizuoka, Japan). All mice were maintained in plastic cages with free access to food and water and housed at $22 \pm 1^\circ\text{C}$ with a 12 h light/dark cycle. Mice were fed a standard laboratory diet (MF; Oriental Yeast Industry, Tokyo, Japan), with the exception of cases wherein atopic dermatitis-like symptoms were induced in hairless mice. The animal study was approved by the committee of the Ethics of Animal Research of Kyoto Pharmaceutical University (approval numbers: 16-12-054 and 17-035).

2.2. Itch models

Three distinct itch models were prepared as follows: (1) Diet-induced atopic dermatitis model (Fujii et al., 2005); after arrival of mice in the animal facility, hairless mice were fed a special diet (HR-AD diet; Norsan, Yokohama, Japan) for 8–12 weeks to fully induce atopic

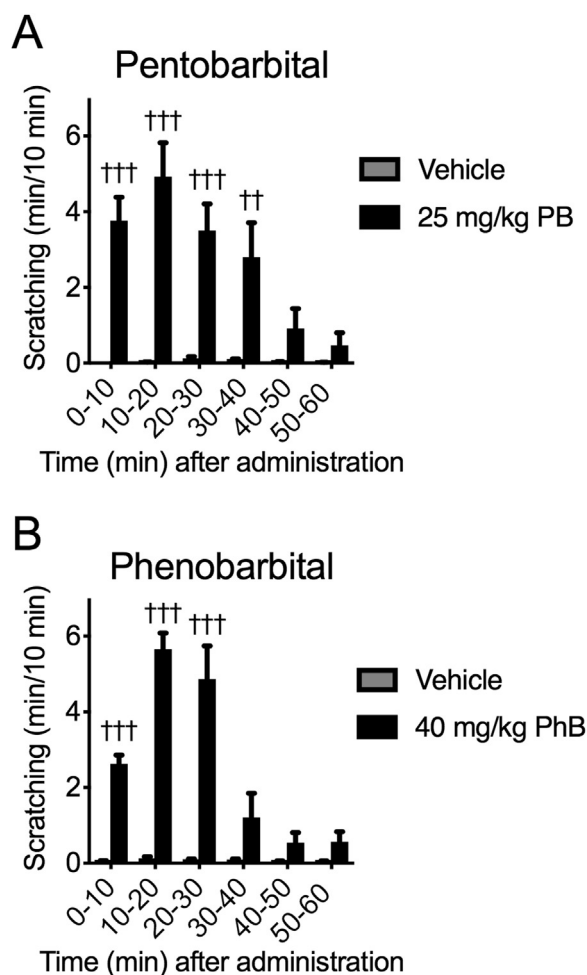


Fig. 2. Time course of scratching behavior after barbiturate administration. Vehicle (saline), pentobarbital (PB, 25 mg/kg, i.p.) (A), or phenobarbital (PhB, 40 mg/kg, i.p.) (B) was administered to special diet-fed atopic mice. Immediately after administration, scratching behavior was measured for 1 h. Each column represents the mean $\pm$ S.E.M. of eight or ten (A), or seven (B) animals. $\dagger\dagger P < 0.01$ and $\dagger\dagger\dagger P < 0.001$, vs. Vehicle.

dermatitis-like symptoms and then used within 1 week. The disease signs were confirmed by gross observation and measurement of skin hydration and transepidermal water loss using Corneometer® CM825 and Tewameter® TM210 (both from Courage + Khazaka Electronic, Cologne, Germany), respectively, as reported previously (Fujii et al., 2005). Normal diet-fed mice were used as a negative control. (2) Naturally occurring atopic dermatitis model (Matsuda et al., 1997); NC/Nga mice spontaneously develop atopic dermatitis-like chronic dermatitis under conventional conditions but not under specific pathogen-free conditions (Matsuda et al., 1997). NC/Nga mice that were healthy or had developed dermatitis (for a minimum of 2 weeks) were purchased from the breeder (Japan SLC) and then used in the experiments after a minimum of 1 week. (3) Pruritogen-induced scratching model (Kuraishi et al., 1995; Fuchibe et al., 2003 with slight modifications); 7-week-old hairless mice were used. Saline or histamine (100 nmol = 11.1 μ g per site) were intradermally (i.d.) injected into the nape of the neck at a volume of 50 μ l using a 29-gauge butterfly needle.

2.3. Behavioral analysis

Hindlimb scratching in mice is thought to be associated with itch sensation (Kuraishi et al., 1995; Shimada and LaMotte, 2008). Thus, in the diet-induced model and the naturally occurring atopic dermatitis model mice, all hindlimb scratching was analyzed regardless of

scratching sites. However, in the pruritogen-induced scratching model mice, only scratching directed to the area around the injection site was analyzed. Prior to observation of scratching, mice were acclimatized for a minimum of 30 min in an observation chamber. Scratching behavior was recorded and then analyzed by playing back the videotape using an in-house counter, as reported previously (Fujii et al., 2005). Briefly, the cumulative duration of scratching was determined by having the observer touch the switch for the duration of the scratching behavior. The time detectable by this instrument was 0.1 s. Using the same counter, we also determined the cumulative duration spent immobile for > 8 s for evaluation of CNS depression by drug administration.

2.4. Drug preparations

For drug preparation, 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX) disodium salt hydrate, histamine dihydrochloride, naltrexone hydrochloride, picrotoxin (all from Sigma-Aldrich, St. Louis, MO, USA), (S)- α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainic acid, muscimol, and phenobarbital sodium (all from Wako Pure Chemical Industries, Osaka, Japan) were dissolved in physiological saline. Pentobarbital sodium (Somnopentyl®; Kyoritsu Seiyaku, Tokyo, Japan) was diluted with physiological saline. (–)-BAY K 8644 (Sigma-Aldrich) was suspended in physiological saline containing 0.5% (v/v) Tween 80. Nifedipine (Sigma-Aldrich) was dissolved in dimethyl sulfoxide and then diluted with physiological saline. Diazepam and triazolam (both from Wako Pure Chemical Industries) were suspended in purified water containing 0.3% (v/v) Tween 80. (+)-Bicuculline (Tokyo Chemical Industries, Tokyo, Japan) was dissolved in 0.1 M HCl, and the pH of the solution was then adjusted to approximately 6 with NaOH, followed by dilution with physiological saline. Each drug was prepared immediately before use.

2.5. Drug administration

Per oral (p.o.) administration was performed using a gavage needle (#5202; Fuchigami Kikai, Kyoto, Japan). Under brief isoflurane (2%) anesthesia, intrathecal (i.t.) injection was performed using a volume of 10 μ l via a lumbar puncture, according to a modified method (Hylden and Wilcox, 1980). Intracisternal (i.c.) injection was performed under conditions of consciousness, as described previously (Ueda et al., 1979). Briefly, the head of the mouse was gently bent and a J-shaped needle (the 27-gauge stainless needle was curved 40 degrees at 3.5 mm from the tip) was inserted into the cleft between the occiput. A volume of 10 μ l aqueous solution was gradually injected into the cisternal magna, which primarily distributed the solution in the area surrounding the cisternal magna and the ventral surface of the brain stem.

In systemic single administration, the dose and route of administration were as follows: pentobarbital (12.5–50 mg/kg, intraperitoneal [i.p.]), phenobarbital (20–80 mg/kg, i.p.), diazepam (1–10 mg/kg, p.o.), triazolam (0.03–1 mg/kg, p.o.), and muscimol (0.5–2 mg/kg, i.p.). When phenobarbital was locally (i.c., i.t., or i.d.) administered, 20 or 40 μ g phenobarbital was injected at a volume of 10 μ l as described above. In the case of combined administration, the dose, route, and timing of administration were as follows: naltrexone (10 and 50 mg/kg) was i.p. administered 30 min before administration of pentobarbital (25 mg/kg, i.p.) or phenobarbital (40 mg/kg, i.p.). Picrotoxin and bicuculline were i.p. administered 5 min after administration of pentobarbital (25 mg/kg, i.p.) or phenobarbital (40 mg/kg, i.p.); the doses of picrotoxin and bicuculline were 5 mg/kg in the case of pentobarbital administration and 3 mg/kg in the case of phenobarbital administration. BAY K 8644 (0.3 and 1 mg/kg) was i.p. administered and immediately followed by administration of pentobarbital (25 mg/kg, i.p.) or phenobarbital (80 mg/kg, i.p.). (S)-AMPA (0.1 and 0.3 nmol/site) or kainic acid (0.1 and 0.3 nmol/site) were i.c. administered after administration of phenobarbital (40 mg/kg, i.p.). When phenobarbital or CNQX were i.c. administered, phenobarbital (10–40 μ g/site) or CNQX

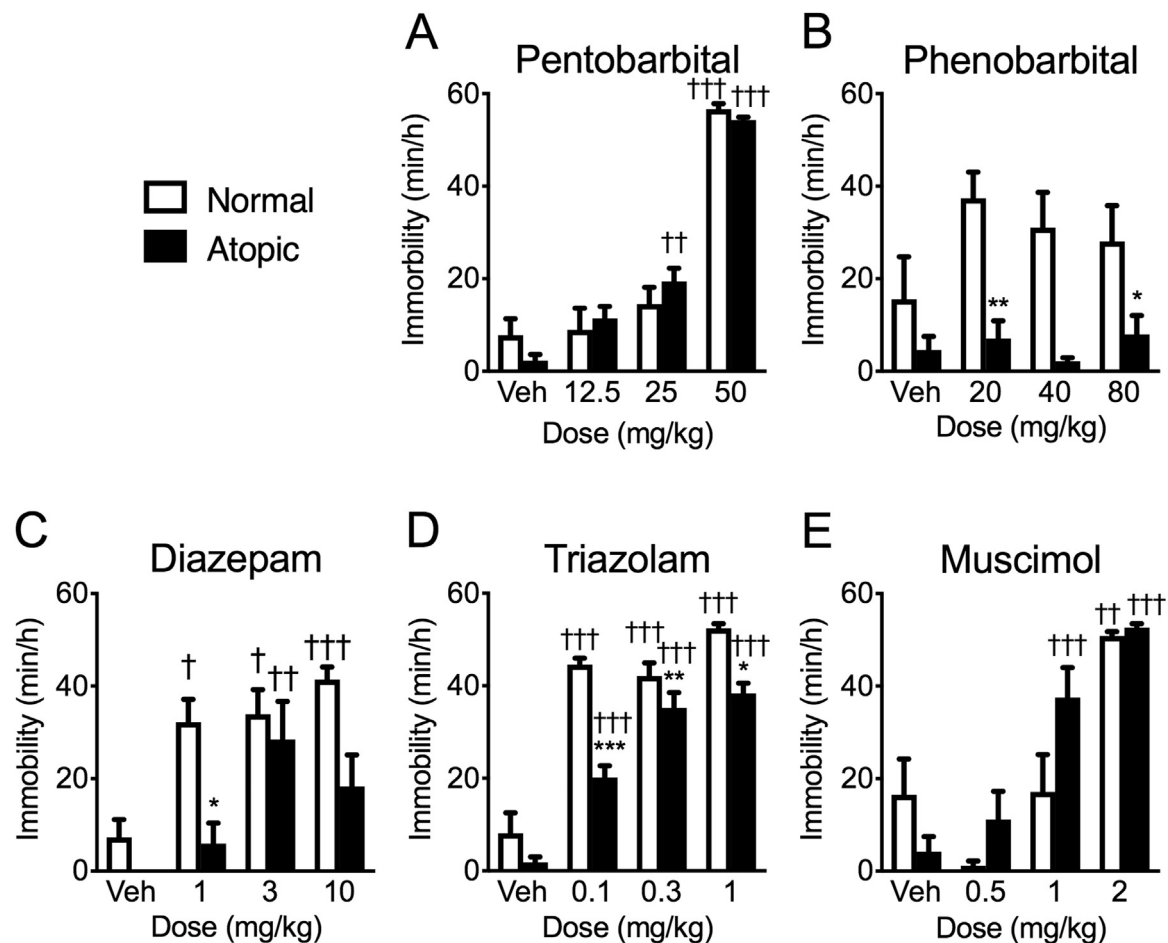


Fig. 3. Effects of barbiturates, benzodiazepines, and muscimol on mobility in normal and atopic mice. (A–E) Vehicle [saline (A, B, and E) and purified water containing 0.3% Tween 80 (C and D)], pentobarbital (12.5, 25, and 50 mg/kg, i.p.) (A), phenobarbital (20, 40, and 80 mg/kg, i.p.) (B), diazepam (1, 3, and 10 mg/kg, p.o.) (C), triazolam (0.1, 0.3, and 1 mg/kg, p.o.) (D), or muscimol (0.5, 1, and 2 mg/kg, i.p.) (E) was administered to mice fed the normal (Normal) or the special (Atopic) diet. Immediately after administration, immobility was measured for 1 h. Each column represents the mean $\pm$ S.E.M. of eight to ten (A), six or seven (B), six or seven (C), eight to eleven (D), or four to seven (E) animals. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, Normal vs. Atopic, and † $P < 0.05$, †† $P < 0.01$ and ††† $P < 0.001$, vs. Vehicle (Veh).

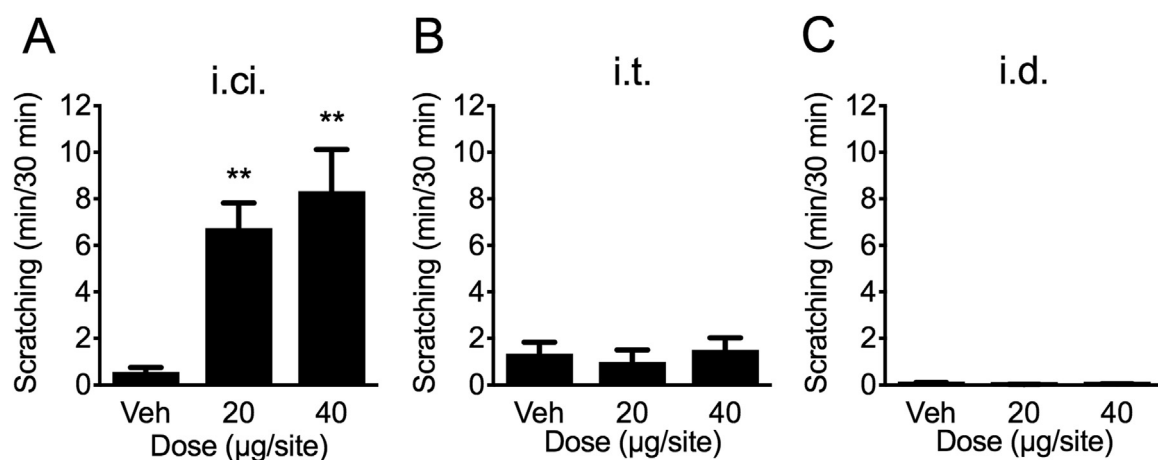


Fig. 4. Scratching responses to intracisternal (i.ci.), intrathecal (i.t.) or intradermal (i.d.) injection of phenobarbital into special diet-fed atopic mice. (A–C) Vehicle (saline, 10 µl/site) or phenobarbital (20 and 40 µg/10 µl/site) was i.ci. (A), i.t. (B), or i.d. (C) administered. Immediately after injection, scratching behavior was measured for 30 min. Each column represents the mean $\pm$ S.E.M. of five (A–C) animals. ** $P < 0.01$, vs. Vehicle (Veh).

(1–4 nmol/site) were injected. Single or mixed solutions of phenobarbital (10 µg/site), CNQX (4 nmol/site), and/or nifedipine (3 pmol/site) were i.ci. administered. Histamine (100 nmol/site) was i.d. injected 5 min after administration of pentobarbital (25 mg/kg, i.p.) or

phenobarbital (40 mg/kg, i.p.). The control group received the vehicle of each drug. All selected doses were based on the results of our preliminary in vivo experiments and those of in vitro experiments reported by others (Kuryshv et al., 2014; French-Mullen et al., 1993; Nardou

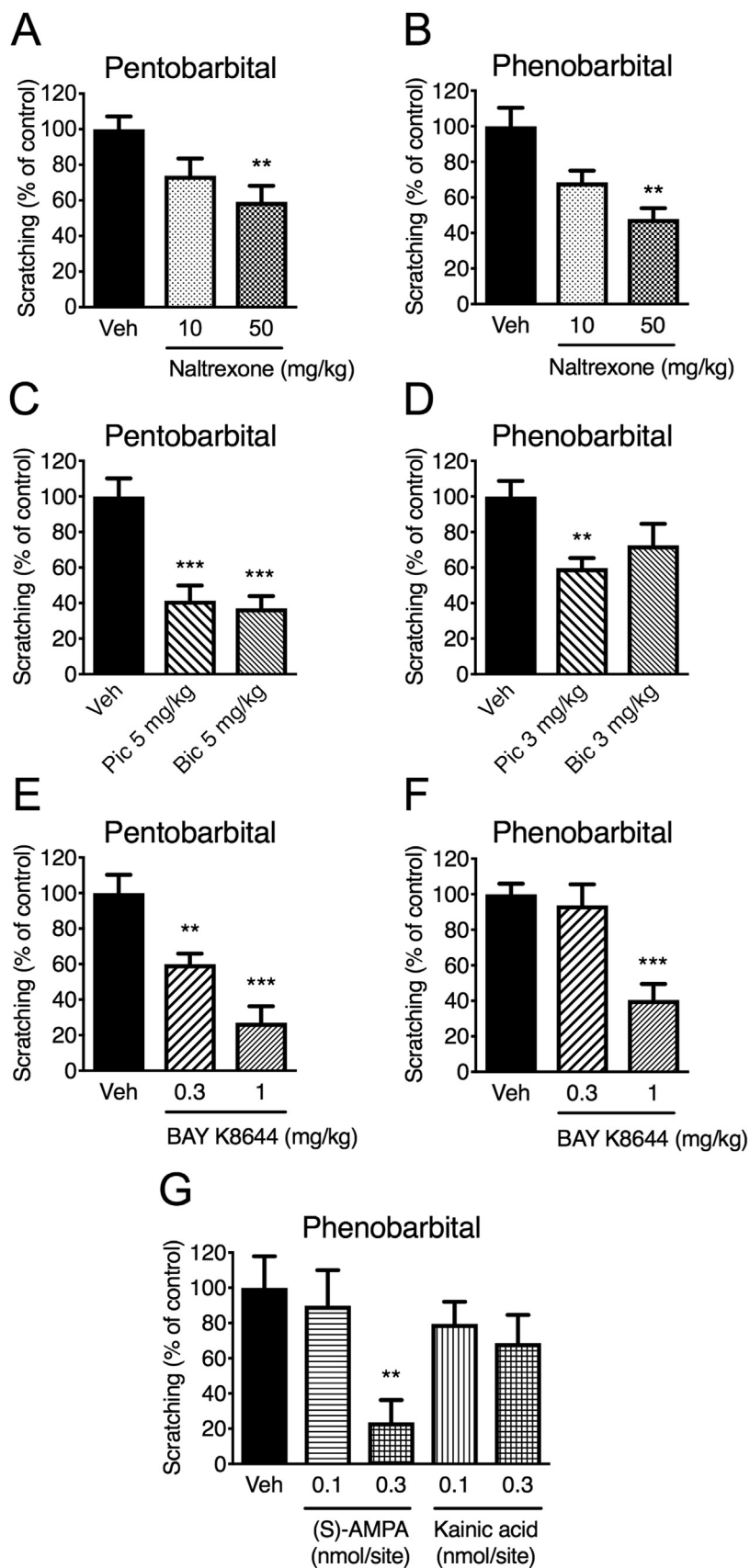


Fig. 5. Effects of several drugs on barbiturate-induced scratching in special diet-fed atopic mice. (A and B) Vehicle (saline) or naltrexone (10 and 50 mg/kg) was i.p. administered 30 min before administration of pentobarbital (25 mg/kg, i.p.) or phenobarbital (40 mg/kg, i.p.). (C and D) Vehicle (saline), picrotoxin (Pic), or bicuculline (Bic) were i.p. administered 5 min after administration of pentobarbital (25 mg/kg, i.p.) or phenobarbital (40 mg/kg, i.p.); the doses of picrotoxin and bicuculline were 5 mg/kg in the case of pentobarbital administration and 3 mg/kg in the case of phenobarbital administration. (E and F) Vehicle (saline containing 0.5% Tween 80), or BAY K 8644 (0.3 and 1 mg/kg) was i.p. administered and immediately followed by administration of pentobarbital (25 mg/kg, i.p.) or phenobarbital (80 mg/kg, i.p.). (G) Vehicle (saline), (S)-AMPA (0.1 and 0.3 nmol/10 μ l/site) or kainic acid (0.1 and 0.3 nmol/10 μ l/site) was i.c.i. administered after administration of phenobarbital (40 mg/kg, i.p.). Immediately after administration, scratching behavior was measured for 1 h. Each column represents the mean $\pm$ S.E.M. of six to 15 (A), six to 14 (B), eight or nine (C), nine (D), nine or ten (E), eight to ten (F), or eight (G) animals. ** $P < 0.01$ and *** $P < 0.001$, vs. Vehicle (Veh).

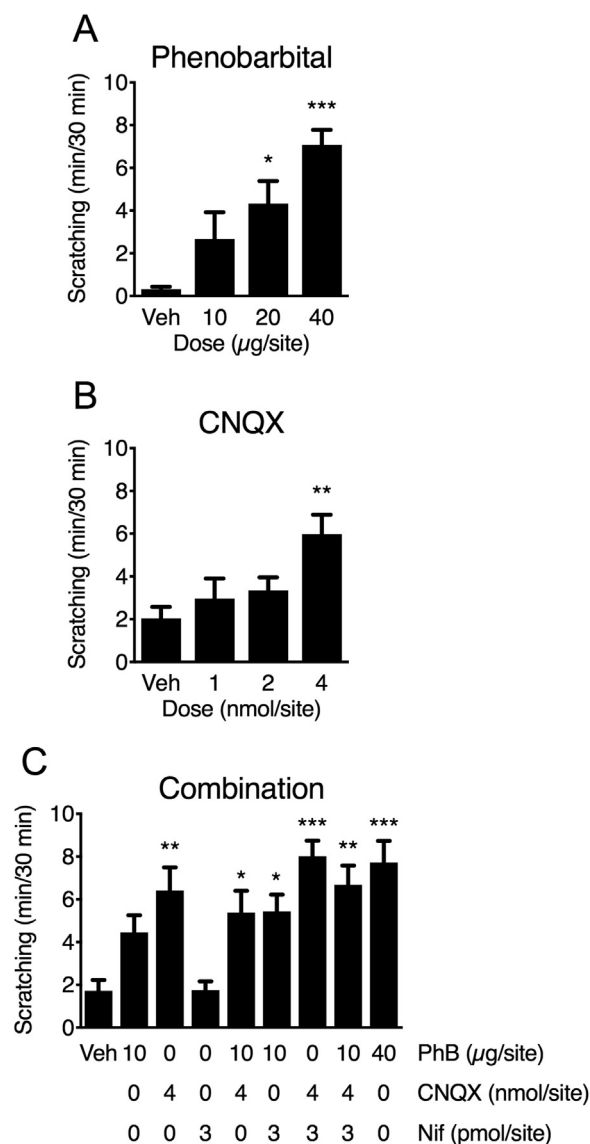


Fig. 6. Scratching responses to single or combined administration of phenobarbital, CNQX, and nifedipine to special diet-fed atopic mice. (A) Vehicle (saline, 10 µl/site) or phenobarbital (10, 20, and 40 µg/10 µl/site) was i.c.i. administered. (B) Vehicle (saline, 10 µl/site) or CNQX (1, 2, and 4 nmol/10 µl/site) was i.c.i. administered. (C) Vehicle (0.003% DMSO-containing saline, 10 µl/site), phenobarbital (PhB, 10 µg/10 µl/site), CNQX (4 nmol/10 µl/site), nifedipine (Nif, 3 pmol/10 µl/site), or their mixed solution was i.c.i. administered. As a positive control, 40 µg/site phenobarbital was i.c.i. injected. Immediately after injection, scratching behavior was measured for 30 min. Each column represents the mean $\pm$ S.E.M. of seven or eight (A), six or seven (B), 11–14 (C) animals. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, vs. Vehicle (Veh).

et al., 2011; Rho et al., 1996).

2.6. Statistical analysis

Data were analyzed using Graphpad Prism (version 7.0; Graphpad Software, San Diego, CA, USA) and are presented as the mean $\pm$ standard error of the mean (S.E.M.). Statistical differences were determined using one-way analysis of variance (ANOVA) with Dunnett's multiple comparison test or two-way ANOVA with Tukey's or Bonferroni's multiple comparison test. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Barbiturates markedly enhance scratching in special diet-fed atopic mice, while benzodiazepines elicit only minor enhancement and muscimol has no effect

We first examined whether classical hypnotics barbiturates, such as pentobarbital and phenobarbital, enhance spontaneous scratching in an atopic dermatitis mouse model. Consistent with our previous result (Fujii et al., 2005), special diet-fed hairless mice exhibited red, wrinkled dry skin resembling that observed in human atopic dermatitis (Fig. 1A bottom), whereas no gross abnormalities were found in normal diet-fed mice (Fig. 1A upper). When pentobarbital or phenobarbital was administered to normal mice, there was no significant increase in the cumulative duration of scratching (Fig. 1B and C). In contrast, in the special diet-fed atopic mice, scratching was dose-dependently and significantly increased by administration of pentobarbital (12.5 and 25 mg/kg) and phenobarbital (20–80 mg/kg) (Fig. 1B and C). The time course of scratching demonstrated that the barbiturate-induced scratching peaked at 10–20 min and had essentially subsided within 60 min (Fig. 2). When pentobarbital was administered at a dose of 50 mg/kg, scratching was not observed due to profound central depressant effects as indicated by a marked increase in the immobility duration (Fig. 1B and Fig. 3A).

We next assessed whether benzodiazepines similarly enhance scratching. Unexpectedly, administration of benzodiazepines diazepam (3 and 10 mg/kg) and triazolam (0.3 and 1 mg/kg) significantly but only slightly increased scratching in atopic mice even with increased immobility (Fig. 1D and E and Fig. 3C and D); the degree of scratching induced by benzodiazepines was considerably less than that induced by barbiturates (Fig. 1B and C). Higher doses of diazepam (30 mg/kg) and triazolam (3 mg/kg) markedly decreased scratching (data not shown). This unexpected result prompted us to further investigate the effect of muscimol, a selective GABA_A receptor agonist, on scratching. Administration of muscimol (1 and 2 mg/kg) significantly decreased scratching in atopic mice, likely due to significant central depressant effects (Fig. 1F and Fig. 3E).

3.2. Barbiturates enhance scratching by a supraspinal mechanism

To determine the site of action of barbiturate-induced scratching in atopic mice, a low dose of phenobarbital was i.c.i., i.t., or i.d. injected. The i.c.i. injection of phenobarbital (20 and 40 µg/site) dose-dependently and significantly increased scratching, whereas the i.t. and i.d. injections at the same dose had no effect (Fig. 4), indicating that barbiturates can act at the supraspinal level to enhance scratching.

3.3. Barbiturate-induced scratching was suppressed not only by GABA_A receptor antagonists but also by an L-type voltage-dependent calcium channel (L-VDCC) agonist and an AMPA glutamate receptor agonist

Next, we pharmacologically characterized barbiturate-induced scratching in atopic mice. Opioid receptor antagonists, such as naloxone and naltrexone, have been demonstrated to suppress various itch responses (Bernstein et al., 1982; Yamaguchi et al., 2001). Thus, we first examined the effect of naltrexone on pentobarbital- and phenobarbital-induced scratching in atopic mice. Naltrexone dose-dependently suppressed scratching by either pentobarbital or phenobarbital, with a significant effect observed at 50 mg/kg (Fig. 5A and B), suggesting that barbiturate-induced scratching is an itch-associated response.

We next examined whether activation of GABA_A receptors is required for barbiturate-induced scratching. When GABA_A receptor antagonists such as picrotoxin and bicuculline were i.p. administered 5 min after administration of pentobarbital (25 mg/kg, i.p.) or phenobarbital (40 mg/kg, i.p.), the scratching response to barbiturates was

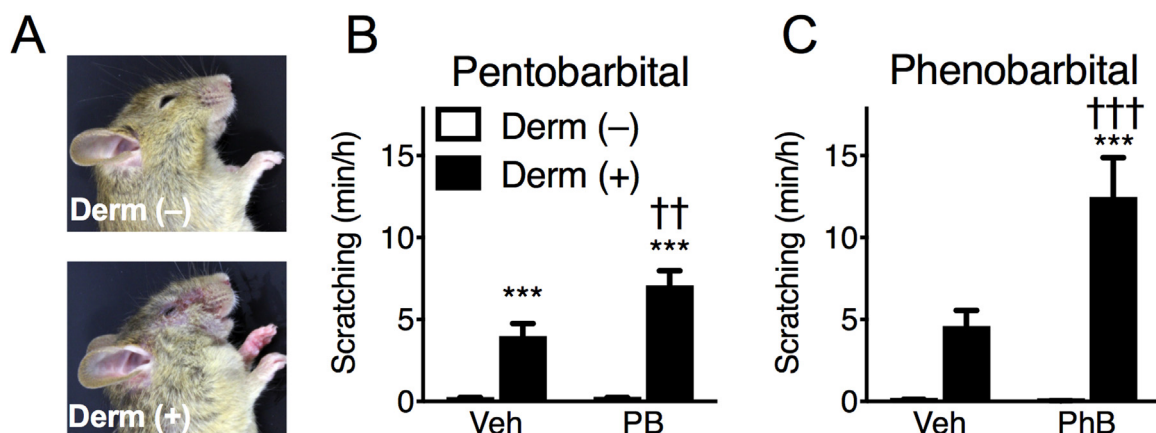


Fig. 7. Effects of barbiturates on scratching behavior in another atopic dermatitis model, NC/Nga mice. (A) Appearance of NC/Nga mice without (–) or with (+) dermatitis (Derm). (B and C) Vehicle (saline), pentobarbital (PB, 25 mg/kg, i.p.) (B) or phenobarbital (PhB, 40 mg/kg, i.p.) (C) was administered to the healthy or diseased NC/Nga mice. Immediately after administration, scratching behavior was measured for 1 h. Each column represents the mean $\pm$ S.E.M. of nine (B and C) animals. *** $P < 0.001$, without vs. with dermatitis, and †† $P < 0.01$ and ††† $P < 0.001$, vs. Vehicle (Veh).

effectively suppressed (Fig. 5C and D). As described above, administration of the selective GABA_A receptor agonist, muscimol, did not enhance scratching (Fig. 1F); this discrepancy between the results of the agonist and the antagonists of GABA_A receptors implies an involvement of CNS targets other than GABA_A receptors in barbiturate-induced scratching. In addition to GABAergic effects, barbiturates are known to inhibit L-VDCC (Dolin et al., 1988; French-Mullen et al., 1993). We thus assessed the effect of the L-VDCC agonist, BAY K 8644, on barbiturate-induced scratching and found that BAY K 8644 dose-dependently and significantly blocked the scratching response to administration of pentobarbital (25 mg/kg, i.p.) and phenobarbital (80 mg/kg, i.p.) (Fig. 5E and F). Barbiturates can also inhibit AMPA/kainate receptors, subtypes of the ionotropic glutamate receptor (Ko et al., 1997; Nardou et al., 2011), and we thus investigated the effect of (S)-AMPA and kainic acid on phenobarbital-induced scratching. When (S)-AMPA (0.1 and 0.3 nmol/site) was i.c.i. injected 10 min after phenobarbital administration (40 mg/kg, i.p.), barbiturate-induced scratching was significantly attenuated, with a significant effect observed at 0.3 nmol per site (Fig. 5G). However, the same dose of kainic acid did not significantly affect barbiturate-induced scratching (Fig. 5G). Although kainic acid also acts as a partial agonist of AMPA receptors, AMPA is shown to act as a high-affinity full agonist (Kew and Kemp, 2005; Sommer and Seeburg, 1992). Therefore, our result suggests a more critical role of AMPA receptors than kainate receptors in barbiturate-induced scratching.

3.4. I.c.i. injected AMPA receptor antagonist alone or in combination with an L-VDCC antagonist sufficiently increases scratching

As barbiturate-induced scratching was blocked by the L-VDCC agonist or the AMPA glutamate receptor agonist (Fig. 5E–G), we next assessed whether single or combined administration of antagonists of L-VDCC or AMPA receptors can enhance scratching in atopic mice. First, further examination of the dose-response effect of i.c.i. injected phenobarbital on scratching revealed that phenobarbital at a dose of 10 μ g/site only tended to increase scratching (Fig. 6A). From preliminary experiments, we could not find any apparent scratching response following an i.c.i. injection of the L-VDCC antagonist, nifedipine (0.3–30 pmol/site), alone (data not shown). However, i.c.i. injection of the AMPA glutamate receptor antagonist, CNQX (1–4 nmol/site), alone caused a dose-dependent increase in scratching with a significant effect at 4 nmol/site (Fig. 6B).

We further examined the effects of combined administration of GABAergic drugs and antagonists of L-VDCC and AMPA receptors. From preliminary experiments, when a mixed solution of CNQX (2 nmol/site) and nifedipine (0.3–30 pmol/site) was i.c.i. injected 3 min after p.o.

administration of diazepam (3 mg/kg), no additional increase in scratching was observed (data not shown). It is widely accepted that the mechanisms underlying the activation of GABA_A receptors by barbiturates and benzodiazepines are different; additionally, the possible involvement of other receptor targets in barbiturate-induced scratching cannot be ruled out. Thus, considering these issues, we decided to use a sub-effective dose of phenobarbital (i.e., 10 μ g/site) in combined administration of CNQX (4 nmol/site) and/or nifedipine (3 pmol/site). Although phenobarbital alone only tended to increase scratching, a significant increase in scratching was observed with the combined administration of phenobarbital and nifedipine (* $P < 0.05$ versus vehicle) (Fig. 6C). CNQX alone significantly increased scratching (** $P < 0.01$ versus vehicle) and there was no further enhancement of scratching by combination with phenobarbital (* $P < 0.05$ versus vehicle) or with phenobarbital and nifedipine (i.e., mix-of-three solution, ** $P < 0.01$ versus vehicle); however, it should be noted that robust scratching was induced by combined administration of CNQX and nifedipine (*** $P < 0.001$ versus vehicle), which was comparable to that by phenobarbital alone at 40 μ g/site (Fig. 6C).

3.5. Barbiturate-induced enhancement of scratching was observed in NC/Nga mice with chronic dermatitis but no enhancement of histamine-induced acute itch was observed in normal healthy mice

Finally, using two additional itch models, we addressed whether barbiturate-induced enhancement of scratching is observed not only in chronic disease conditions but also in acute itch in healthy conditions. NC/Nga mice chronically experiencing dermatitis exhibited apparent spontaneous scratching, which was significantly enhanced by administration of either pentobarbital (25 mg/kg, i.p., †† $P < 0.01$ versus vehicle) or phenobarbital (40 mg/kg, i.p., ††† $P < 0.001$ versus vehicle) (Fig. 7). However, the same doses of pentobarbital and phenobarbital did not affect acute scratching induced by i.d. injection of histamine into the skin of normal hairless mice (Fig. 8).

4. Discussion

In this study, we found that systemic administration of either barbiturates (pentobarbital and phenobarbital) or benzodiazepines (diazepam and triazolam) significantly enhanced scratching in diet-induced atopic dermatitis model mice, although the enhancement of scratching by benzodiazepines was considerably less than that induced by barbiturates. Barbiturates were previously used as hypnotics for patients with insomnia, although they have now been largely replaced by benzodiazepines due to the lower risk of drug dependence and overdose

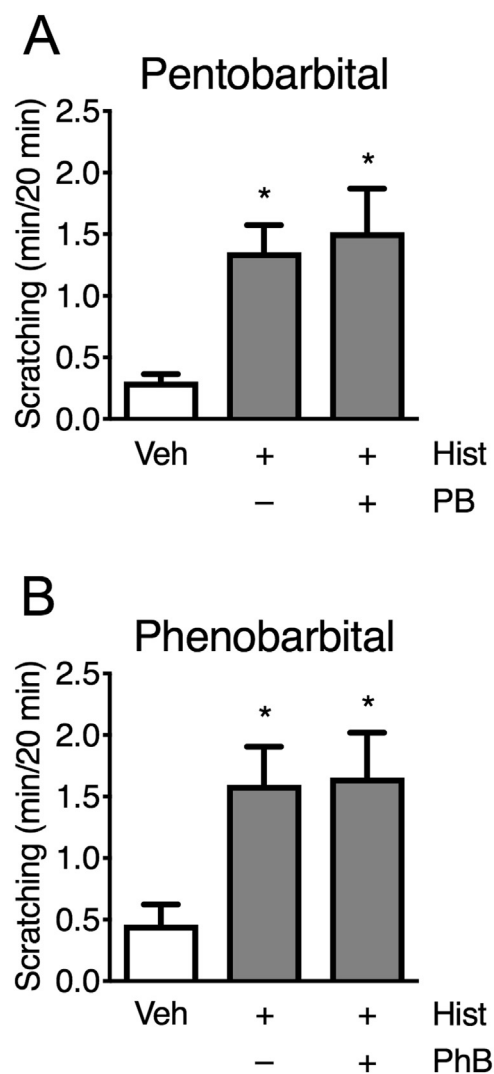


Fig. 8. Effects of barbiturates on histamine-induced scratching behavior in normal healthy mice. (A and B) Vehicle (saline), pentobarbital (PB, 25 mg/kg, i.p.) (A) or phenobarbital (PhB, 40 mg/kg, i.p.) (B) was administered to normal hairless mice, and 5 min later, histamine (Hist, 100 nmol/site) was i.d. injected to the nape of the neck. Immediately after histamine injection, scratching of the injection site was measured for 20 min. Each column represents the mean $\pm$ S.E.M. of five (A) or six or seven (B) animals. * $P < 0.05$, vs. Vehicle (Veh).

(Löscher and Rogawski, 2012). Although the barbiturate doses that enhanced scratching in this study seem very high, the equivalent human doses (Reagan-Shaw et al., 2008) are almost hypnotic. Indeed, the present doses of barbiturates have been demonstrated to produce hypnotic or sub-hypnotic effects in mice (Abdel Gawad et al., 2012; Zhao et al., 2006). Furthermore, a neuropharmacological study has demonstrated that the sedative (or hypnotic) properties of barbiturates involve actions on specific neuronal pathways that contribute to natural sleep (Nelson et al., 2002). Therefore, these pieces of evidence suggest that the enhancement of scratching in the mouse model, in particular that induced by barbiturates, partly mimics nocturnal pruritus in human atopic dermatitis. However, it should be noted that drug-induced sleep is not equivalent to natural physiological sleep. Therefore, future studies should examine the effects of natural sleep and arousal on scratching in chronic itch cases with electroencephalography.

Our local injection studies indicate that barbiturate-induced scratching is mediated via a supraspinal action. This finding is, to an extent, expected but has important implications for elucidating an

unidentified supraspinal mechanism contributing to the induction and modulation of itch.

The present pharmacological study suggests that both GABA_A receptor activation and AMPA receptor and L-VDCC inhibition contribute to barbiturate-induced scratching. This conclusion is consistent with the fact that, although barbiturates are generally thought to exert effects primarily via GABA_A receptors, they have many additional targets including L-VDCC and AMPA glutamate receptors (Löscher and Rogawski, 2012). The result indicating that i.c.v. injected CNQX alone significantly enhanced scratching suggests that the inhibitory effects of AMPA receptors play the most critical role in barbiturate-induced scratching. This conclusion is also supported by the fact that GABAergic benzodiazepines, which only weakly enhanced scratching in this study, can inhibit L-VDCC but not AMPA receptors (Earl and Tietz, 2011; Nardou et al., 2011). However, the mechanism by which GABA_A receptor activation and L-VDCC inhibition synergistically affect glutaminergic AMPA pathways to enhance scratching remains unclear.

In addition to the possible involvement of AMPA receptors and L-VDCC, GABA_A receptor heterogeneity may contribute to barbiturate-induced scratching. GABA_A receptors can be roughly divided into two subtypes based on the presence of γ or δ subunits. The γ - and δ -containing receptors are mainly located synaptically and extrasynaptically, respectively, and thereby contribute to phasic and tonic GABA-mediated inhibitory transmission, respectively (Winsky-Sommerer, 2009). Although benzodiazepines specifically bind the γ -containing receptors, barbiturates bind the β subunits present in both GABA_A receptor subtypes (Saxena and Macdonald, 1994). Several lines of evidence have shown that ethanol also acts on both receptor subtypes (Santhakumar et al., 2007). As for muscimol, although it is generally regarded as a GABA_A receptor pan-agonist, its in vivo effects are reportedly mediated preferentially through a small population of GABA_A receptors containing δ subunits (Chandra et al., 2010; Korpi et al., 2002). Given these between-drugs differences in effects on GABA_A receptors, activation of both GABA_A receptor subtypes may be necessary to fully induce scratching in atopic mice. However, further studies involving γ or δ subunit knockout mice are necessary to test this hypothesis, and the effects of combinations of benzodiazepines, muscimol, and CNQX (and/or nifedipine) on scratching should also be examined.

We found that barbiturate administration significantly enhanced scratching in NC/Nga mice suffering from chronic dermatitis for several weeks, while it did not affect histamine-induced acute itch in healthy mice. Our preliminary experiment also indicated that scratching was slightly (but not significantly) enhanced by pentobarbital administration in a “sub”-chronic itch model induced by repeated (twice daily for 5 days) treatment with acetone/ether and water (Fujii M., unpublished data). These results suggest that such enhancement of scratching is observed specifically in highly chronic itch conditions. Although the mechanism remains unclear, we hypothesize that suppression of an inhibitory itch system in the brain is involved. Several studies suggest that periaqueductal gray plays a critical role in modulating itch as well as pain (Carstens, 1997; Mochizuki et al., 2003; Papoiu et al., 2013). Notably, it has been reported that electrical stimulation-induced neural activity in the periaqueductal gray was attenuated by pentobarbital or the L-VDCC antagonist verapamil (Schenberg et al., 2000; Smith et al., 1992). Furthermore, glutaminergic neurons descending from the periaqueductal gray to the rostral ventromedial medulla or the locus coeruleus and the AMPA glutamate receptors have been demonstrated to significantly contribute to pain modulation (Ennis et al., 1992; Lau and Vaughan, 2014; Spinella et al., 1996). When applying the mechanism of pain modulation, and if a descending itch inhibitory system is strengthened to counteract chronic severe itch, it is possible that barbiturates (and possibly certain sleep conditions) attenuate the itch modulation, resulting in the apparent enhancement of scratching. However, further studies using traditional microinjection or novel optogenetic approaches are warranted to test this hypothesis.

This study has several limitations. Our experiments were performed

in animal models, and our findings can therefore not be simply extrapolated to clinical situations. Interestingly, however, in a few clinical cases, sedative doses of barbiturates have been reported to increase itch-associated scratching (Muston et al., 1979), whereas benzodiazepines have been reported to either suppress or not affect nocturnal scratching in dermatitis patients (Ebata et al., 1998; Krause and Shuster, 1983), which is consistent with our findings. However, so far, no sufficient clinical evidence regarding the effects of barbiturates on pruritus is available because barbiturates are no longer used as hypnotics.

5. Conclusion

Our data indicate that barbiturates markedly enhance itch-associated scratching in atopic mice, and suggest that a synergistic effect among AMPA receptor inhibition, GABA_A receptor activation, and L-VDCC inhibition in the brain mediates barbiturate-induced scratching. Although the results could not exclude the possibility that mechanisms other than the CNS depressant effects may be involved in nocturnal pruritus in atopic dermatitis (Lavery et al., 2016; Patel et al., 2007), our observation may provide novel insights into the understanding of nocturnal pruritus and supraspinal itch mechanisms in chronic pruritic conditions. A better understanding of the mechanism of action would lead to the development of therapeutic approaches for itch in chronic pruritic diseases including atopic dermatitis.

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