Sleep-Time Variation for Ethanol and the Hypnotic Drugs Tribromoethanol, Urethane, Pentobarbital, and Propofol within Outbred ICR Mice

Tsutomu KOIZUMI¹⁾, Hideyuki MAEDA¹⁾, and Kyoji HIOKI²⁾

¹⁾Laboratory Animal Center, Fukui Medical University, 13-1 Shimoaitsu, Matsuoka-cho, Fukui 910-1193 and ²⁾Central Institute for Experimental Animals, 1430 Nogawa, Miyamae-ku, Kawasaki-shi 216-0001, Japan

Abstract: To evaluate the phenotypic variation within a commercial outbred mouse stock, we examined sleep-time (or duration of loss of righting reflex) of outbred ICR mice after i.p. injection of ethanol (4.0 g/kg of body weight), urethane (1.3 g), tribromoethanol (250 mg), and pentobarbital (60 mg), and after i.v. injection of propofol (30 mg). We observed high-grade individual differences in sleep-time that ranged from 0 to 179 min, 83.1 ± 4.3 (mean and SEM of 100 mice) for ethanol; 0 to 169 min, 64.5 ± 3.1 for pentobarbital; 0 to 160 min, 36.6 ± 3.6 for urethane; 0 to 120 min, 21.5 ± 2.2 for tribromoethanol; and 3 to 20.5 min, 7.1 ± 0.3 for propofol. This extensive phenotypic variance within the outbred stock was as great as the variation reported among inbred strains or selected lines, and the varied susceptibility within the colony was inherited by Jcl:ICR-derived inbred strains IAI, ICT, IPI, and IQI. The range of sleep-time variance for ethanol, pentobarbital, urethane, tribromoethanol, and propofol within four-way cross hybrid Jcl:MCH(ICR) mice was 86.6%, 63.3%, 124%, 61.0%, and 53.1% that of outbred Jcl:ICR mice, respectively. The present study indicates that phenotypic variance within an outbred Jcl:ICR stock was at high risk for susceptibility to the drugs that depress the central nervous system and that Jcl:ICR-derived inbreds may be an excellent source of animal models for studying the anesthesia gene.

Key words: hypnotic sleep, ICR-derived inbred strain, outbred ICR mice

Introduction

Various kinds of substances, including rare gasses, alkanes, ketone, ether, alcohol, halogenated hydrocarbons, and steroids elicit general anesthesia when given in a sufficient dose [7]. Though molecular pharmacol-

ogical analyses have revealed that general anesthetics can affect an almost infinite variety of molecular targets [7, 8, 16] in the central nervous system, it is still uncertain how and where general anesthetics act [8]. On the other hand, individual differences (or strain differences) in susceptibility to general anesthetics are well

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Address corresponding: T. Koizumi, Laboratory Animal Center, Fukui Medical University, 13-1 Shimoaitsu, Matsuoka-cho, Fukui 910-1193, Japan

known, and selective breeding from genetically heterogeneous stock based on susceptibility or resistibility has created mouse and rat strains with different sensitivities to ethanol [6, 21], nitrous oxide [15], and diazepam sleep [11]. Quantitative trait locus (QTL) analysis for the genes concerning sensitivity to general anesthesia is thought to be one of the promising genetic approaches to understanding the mechanism of general anesthesia [2, 3, 22]. Analysis using genetically engineered (e.g., transgenic, gene knockout) mice that harbor very specific alterations in single genes of interest is also a promising approach [12]. QTL analysis has been reported only for ethanol [18, 19] and propofol sleep [26]. This is partly because the mouse or rat selected with one drug (e.g., ethanol) has no difference in susceptibility to other drugs. For example, there is little or no difference in susceptibility to pentobarbital and tribromoethanol between long-sleep (LS) and short-sleep (SS) mice, which were selected based on their ethanol sleep sensitivity [1, 5, 24], and the same is true of susceptibility to pentobarbital between high susceptible (HI) and low susceptible (LO) mice selectively bred for resistance or susceptibility to nitrous oxide anesthesia [16]. In QTL analysis, many samples are required if the difference in susceptibility between parental strains is small, or many genes in which the individual effect is small are involved in the difference. Therefore, several animal models are required for the QTL analysis of the anesthesia gene.

In the present study, we examined the sensitivity of Jcl:ICR stock to ethanol and anesthesia because several inbred strains have been established from this colony [14]. We observed a high degree of individual differences in sleep-time in Jcl:ICR mice, and a wide degree of phenotypic variance within the outbred stocks was inherited by Jcl:ICR-derived inbred IAI, ICT, IPI, and IQI mice.

Materials and Methods

Animals: Six-week old outbred Jcl:ICR and four-way cross hybrid Jcl:MCH(ICR) (MCH(ICR)) mice were purchased from CLEA Japan Inc., (Tokyo). MCH(ICR) mice were produced by four-way cross of Jcl:ICR-derived inbred strains IAI/Jcl, ICT/Jcl, IPI/Jcl, and IQI/Jcl, as shown in Fig. 1. Inbred IAI/Jcl, ICT/Jcl, IPI/Jcl, and IQI/Jcl mice were bred at our laboratory. Mice

Fig. 1. Mating system (four-way cross) for hybrid Jcl:MCH(ICR) mice.

were housed in autoclaved plastic cages with soft woodchip bedding in an air-conditioned room (temperature $23 \pm 2^\circ\text{C}$, humidity 50–70%), artificially illuminated daily from 06:00 to 20:00 hr, and were fed mouse chow (CE-2; CLEA Japan Inc., Tokyo, Japan) and water *ad libitum*. All procedures of this study were approved by the Institutional Animal Care and Use Committee of Fukui Medical University.

Drug preparation and administration: Tribromoethanol 100% stock solution was prepared as described elsewhere [23]. Briefly, 10 g of 2,2,2-tribromoethanol (Aldrich Chemical Co., Milwaukee, WI, USA) was dissolved in 10 ml of tert-amylalcohol (Nacali Tesque, Inc., Kyoto, Japan). This stock solution was diluted to 2.5% with sterile distilled water, stored at 4°C in the dark, and intraperitoneally administered at a rate of 0.1 ml/10 g body weight (250 mg/kg of body weight [20]). Ethanol was diluted with physiological saline to a final concentration of 20% w/v and intraperitoneally administered at a rate of 0.25 ml/10 g body weight (4.0 g/kg [18]). Urethane (Sigma Chemical, St. Louis, MO, USA) was dissolved 100 mg/ml in physiological saline and intraperitoneally administered at a rate of 0.13 ml/10 g (1.3 g/kg [20]). Pentobarbital (Nembutal 50 mg/ml solution, Dynabot, Tokyo, Japan) was diluted 1:20 with physiological saline and administered at a rate of 0.24 ml/10 g (60 mg/kg [20]) intraperitoneally. Propofol (Diprivan 1%, Zeneca Pharmaceutical Co., Osaka, Japan) was directly used without further dilution and injected at a rate of 0.1 μl /10 g body weight (30 mg/kg [26]) into an orbital vein as described previously [26].

Sleep-time: One-hundred Jcl:ICR or MCH(ICR) mice were divided into five groups of 20 mice, 10 females and 10 males. The procedures for the sleep-time tests

were performed in a temperature-controlled (23 to 25°C) room as previously described [5]. Sleep-time studies were started at the age of seven weeks. Mice were injected with propofol, tribromoethanol, urethane, ethanol and pentobarbital in that order at 7- to 10-day intervals, and the time between loss of righting reflex (LORR) and regain of righting reflex (RORR) was defined as sleep-time. Animals were judged to have LORR when they could not right themselves twice within a 30-sec period after being placed on their backs on a stainless steel laboratory table. Mice were judged to have regained the righting reflex when they could right themselves twice within a 30-sec period. If mice failed to lose the righting response within 20 min after drug administration, a sleep time of zero was recorded.

Data were analyzed with one-way analysis of variance (ANOVA) or two-way ANOVA and an alpha level of 0.05 was adopted for significance testing.

Results

Ethanol sleep: Ethanol sleep-time of outbred Jcl:ICR mice ranged from 0 to 179 min. There was no sex or group difference in sleep-time variation (data not shown), and the mean sleep-time of 100 Jcl:ICR mice was 83.1 ± 4.3 min ($\pm$ SEM). Three strains, ICT, IPI, and IQI, inherited the ethanol sleep susceptible trait while IAI inherited the ethanol sleep resistant trait. The sleep-time in MCH(ICR) mice ranged from 48 to 203 min, and the mean sleep time was 32.5 min longer than that of Jcl:ICR mice (Fig. 2). The same variation was observed in five experimental groups of MCH(ICR) mice.

Pentobarbital sleep: There was no sex or group difference in sleep-time variation in pentobarbital sleep-time of outbred Jcl:ICR mice, which varied widely. The mean sleep-time of 100 Jcl:ICR mice was 65.4 ± 3.1 min and ranged from 0 to 169 min. The inbred IAI strain inherited the sleep susceptible trait, while the IQI inherited a relatively resistant trait. A highly resistant trait for pentobarbital sleep was not observed among the four inbred strains. The mean sleep-time of MCH(ICR) was 15.8 min longer than that of outbred Jcl:ICR mice (Fig. 3).

Urethane sleep: Susceptibility to urethane anesthesia of Jcl:ICR mice was widely different within the stock. There was no difference in sleep-time variance among experimental groups, however, the sleep-time of males (29.3 ± 3.3 min) and that of females (43.7 ± 6.2 min)

Fig. 2. Ethanol sleep-time. Mean and SEM of 16 to 20 mice for each inbred strain (left part). Box-and-whisker plots of the sleep-time data of 100 mice for each outbred Jcl:ICR and MCH(ICR) strain (right part). The ends of boxes are the 25th and 75th quantiles and horizontal lines represent 10th, 50th and 90th quantiles, respectively.

were significantly different ($p < 0.05$). Therefore the data of male and female mice were analyzed separately. Sex difference was observed in ICT and IQI mice, while no significant difference was observed in IAI, IPI, and MCH(ICR) mice. Members of an inbred IAI strain inherited a highly resistant trait for urethane anesthesia, while IPI mice inherited a very susceptible trait. No significant difference was observed between Jcl:ICR and MCH(ICR) mice (Fig. 4).

Tribromoethanol sleep: Susceptibility to tribromoethanol anesthesia of Jcl:ICR mice was equal among experimental groups but widely different between male and female mice ($P < 0.0001$). Females showed more susceptibility than males. However, sex difference was not observed in IAI, IQI, or MCH(ICR) mice. An inbred IAI strain inherited a highly susceptible trait for tribromoethanol anesthesia, while IQI mice inherited a very susceptible trait (Fig. 5). In MCH(ICR) mice, the sleep-time of two experiment groups was significantly different from that of the other three groups (data not shown).

Fig. 3. Pentobarbital sleep-time. Mean and SEM of 16 to 20 mice for each inbred strain (left part). Box-and-whisker plots of the sleep-time data of 100 mice for each outbred Jcl:ICR and MCH(ICR) strain (right part). The ends of boxes are the 25th and 75th quantiles and horizontal lines represent 10th, 50th and 90th quantiles, respectively.

Propofol sleep: Sleep-time of outbred Jcl:ICR mice varied widely, ranging from 3 to 20.5 min, while no group or sex difference was observed. An inbred IPI strain inherited a sleep susceptible trait, while IAI, ICT, and IQI strains inherited intermediate sensitivity and a highly resistant trait observed in outbred Jcl:ICR mice was not inherited among the four inbred strains. The sleep-time of MCH(ICR) was slightly longer than that of Jcl:ICR mice (Fig. 6), though the data were statistically not significant.

Discussion

There have been few reports of genetic variation within an outbred colony of mice. Rice and O'Brien [25] studied genetic variation at 46 loci in three outbred Swiss mouse colonies and observed polymorphism at 6 (13.0%), 8 (17.4%), and 9 (19.6%) loci, respectively. Kato [14] studied genetic variation at 22 loci among 30 inbred strains, which were established from outbred Jcl:ICR

Fig. 4. Urethane sleep-time. Mean and SEM of 8 to 10 mice for each sex of inbred strain (left part). Box-and-whisker plots of the data of 50 mice for each sex and strain of outbred Jcl:ICR and MCH(ICR) strain (right part). The ends of boxes are the 25th and 75th quantiles and horizontal lines represent 10th, 50th and 90th quantiles, respectively.

stock, and found polymorphism in 15 (68.2%) loci. Cui *et al.* [4] examined 10 loci in five Swiss mouse colonies maintained in Australia and found 30% to 80% polymorphism in a small number of samples, within each colony. However, Festing [9] disagrees with the conclusion that outbred Swiss mice hold a considerable degree of genetic variation within a colony because it was hard to estimate quantitatively in the above studies since the loci chosen are known to be polymorphic among mice, rather than randomly selected. Furthermore, even if there is considerable genetic variation within an outbred stock, the phenotypic variation in a small number of samples of an outbred colony may not be large in comparison with the phenotypic variation that may be observed between two different inbred strains.

In the present study, we observed large phenotypic variation within the outbred Jcl:ICR mice even when a small number of mice (10 mice of each sex) was examined. In ethanol sleep, variation within Jcl:ICR stock was at the same level as was observed between SS and LS mice, which are selectively bred from 8-way cross

Fig. 5. Tribromoethanol sleep-time. Mean and SEM of 8 to 10 mice for each sex of inbred strain (left part). Box-and-whisker plots of the data of 50 mice for each sex and strain of outbred Jcl:ICR and MCH(ICR) strain (right part). The ends of boxes are the 25th and 75th quantiles and horizontal lines represent 10th, 50th and 90th quantiles, respectively.

Fig. 6. Propofol sleep-time. Mean and SEM of 16 to 20 mice for each inbred strain (left part). Box-and-whisker plots of the sleep-time data of 100 mice for each outbred Jcl:ICR and MCH(ICR) strain (right part). The ends of box are the 25th and 75th quantiles and horizontal lines represent 10th, 50th and 90th quantiles, respectively.

genetically heterogeneous mice. The mean sleep-time of LS and SS mice after 4.1 g/kg of body weight i.p injection was 182.6 and 6.6 min, respectively [18]. The degree of variation in pentobarbital sleep-time of outbred Jcl:ICR mice in the present study was as much as that reported in one among 23 inbred and congenic strains [17]. Moreover, the fact that a considerable number of mice resisted pentobarbital sleep at the dose of 60 mg/kg (Fig. 3) indicates the possibility of establishing a highly pentobarbital sleep resistant strain from outbred Jcl:ICR stock. The variation of outbred Jcl:ICR mice in urethane sleep-time was at the same level as the reported difference between LS and SS mice [20]. In tribromoethanol sleep, a wide range of variation was observed within Jcl:ICR mice, while no obvious strain difference has been reported among inbred strains, and sleep time reported for LS mice was almost equal to those of SS mice [20]. Propofol sleep-time differences of Jcl:ICR mice were equal to that observed between C57BL/6 and 129 strains [13] and between LS and SS mice [26].

According to Kato [14], 33 lines of inbred strains were established from outbred Jcl:ICR stock in the 1970s by Yoshimura and colleagues at the Central Institute for

Experimental Animals. Unfortunately, 17 lines became extinct between 21 to 35 generations. Of 22 loci examined, polymorphism was observed at 8 loci (36.3%) among IAI, ICT, IPI, and IQI mice, the parental strains of MCH (ICR) mice. In the present study, phenotypic variations within four Jcl:ICR-derived inbred strains and MCH (ICR) mice were also examined. Festing [10] speculated that if several inbred strains are established from a single outbred colony, the range of phenotype among the inbred strains will usually be substantially greater than the range found in original outbred stocks. In the present study, variation in response to urethane and tribromoethanol within four inbred strains was greater than that found in Jcl:ICR stock, but variation in response to propofol, ethanol, and pentobarbital was smaller than that found in original stocks. This is mainly because highly resistant traits for these drugs were not found in the four ICR-derived inbred strains.

In the present study, a mouse strain-dependent sex difference was observed in tribromoethanol and urethane sleep-times. Such genotype-dependent expression of sex difference is also observed in pentobarbital sleep [17],

though no obvious sex difference was observed among the mice used in the present study. Interaction of the sex chromosome gene and the gene(s) responsible for the pharmacokinetic or pharmacodynamic processes of hypnotic drugs may explain the genetic dependence of the sexual differences observed in the present study.

Our present study clearly shows that phenotypic variation within commercial outbred ICR mice is not small as had been speculated, but rather is large, at least for the drugs that depress the central nervous system. The variety in susceptibility within outbred Jcl:ICR was inherited by the Jcl:ICR-derived inbred strains IAI, ICT, IPI, and IQI.

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