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NOTE

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Combined medetomidine, midazolam, and butorphanol anesthesia in mice has a central stress-relieving effect similar to that of isoflurane anesthesia

Makoto YOKOSUKA[1)], Mako OSAKA[2,3)], Atsushi TOHEI[2)]*

[1)]Laboratory of Comparative Medicine, Nippon Veterinary and Life Science University, Tokyo, Japan
[2)]Laboratory of Experimental Animal Science, Nippon Veterinary and Life Science University, Tokyo, Japan
[3)]Shionogi Techno Advance Research Co., Ltd., Osaka, Japan

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ABSTRACT. Although the combination of medetomidine, midazolam, and butorphanol (Me/Mi/Bu) is a commonly used surgical anesthetic for small laboratory animals, the effects of Me/Mi/Bu on the central nervous system remain to be confirmed, and some researchers have questioned the use of Me/Mi/Bu as a surgical anesthetic. Herein we employed cFos-immunohistochemistry to assess the stress-relieving effects of Me/Mi/Bu and isoflurane on the murine brain in response to restraint stress. The results demonstrated that the number of cFos-immunopositive cells in the paraventricular nucleus was significantly lower in the mice anesthetized with Me/Mi/Bu or isoflurane compared to those that were not anesthetized. These findings suggest that Me/Mi/Bu exerts an effect on the brain that is similar to that of isoflurane in alleviating the response to surgical procedures.

KEYWORDS: anesthesia, isoflurane, mice, paraventricular nucleus of the hypothalamus, restraint-stress

Isoflurane, an inhaled anesthetic, is a commonly used agent for surgical anesthesia in laboratory animals [1, 8]. Small laboratory animals such as rats and mice can also be anesthetized via an intraperitoneal injection of a mixture of ketamine and other drugs, including xylazine and medetomidine [1, 5, 8]. As in western countries, ketamine is classified as a controlled substance in Japan, and drug-free the combination of medetomidine, midazolam, and butorphanol (Me/Mi/Bu) is thus a prevalent alternative [7, 9, 10, 17–19]. The use of Me/Mi/Bu is advantageous for the following reasons: (*i*) it does not necessitate the use of a dedicated vaporizer (as does the inhalation anesthetic isoflurane), thus resulting in reduced costs, and (*ii*) it allows for the rapid induction of arousal through the administration of atipamezole, which acts as an antagonist to medetomidine. This enables experimenters to control the timing of arousal. Conversely, the constituents of Me/Mi/Bu are all pharmaceutical agents used as sedatives and analgesics in veterinary medicine.

Although the anesthetic efficacy of Me/Mi/Bu has been substantiated through the suppression of peripheral pain [7, 9, 10, 19], its anesthetic impact on the central nervous system, particularly the brain, has yet to be directly validated through the use of electroencephalogram (EEG) data or alternative methodologies [13]. Consequently, despite the inclusion of Me/Mi/Bu in textbooks on the anesthesia of laboratory animals [3], some researchers have expressed reservations about its use as a surgical anesthetic. To address this issue, it is essential to ascertain whether the action of Me/Mi/Bu is directly correlated with neural activity in the brain, akin to the anesthetics commonly employed as surgical anesthetics, such as isoflurane [23].

We conducted the present study to determine whether anesthesia with Me/Mi/Bu directory effects on neural activity in the brain in a manner analogous to that of isoflurane. To this end, we investigated the neural inhibition effects of the Me/Mi/Bu and isoflurane for the paraventricular nucleus of the hypothalamus (PVN), which is intimately associated with the perception of mechanical and mental stress [11, 20, 21].

We used 8- to 9-week-old male ICR mice (Tokyo Experimental Animals, Tokyo, Japan) for the experiments. All animals were individually housed at 23–25°C, 40–60% humidity, with a 12-hr light-dark cycle (light period: 7:00–19:00) in the animal room permanently established with institutional approval at the Laboratory of Comparative Animal Medicine, Nippon Veterinary and Life Science University. Wooden shavings (White Flake; Oriental Yeast Co., Tokyo, Japan) were used as bedding, and laboratory animal solid food (NMF; Oriental Yeast Co.) was provided *ad libitum* together with tap water for drinking. The study was conducted after

*Correspondence to: Tohei A: tohei@nvlu.ac.jp, Laboratory of Experimental Animal Science, Nippon Veterinary and Life Science University, 1-7-1 Kyonan, Musashino, Tokyo180-8602, Japan

approval was obtained in accord with the University's internal regulations (approval no. 2023S-24). There are no conflicts of interest to disclose.

To reduce stress due to experimental procedures such as drug administration and to minimize elevated cFos protein immunoreactivity (cFos-IR) caused by procedures other than restraint stress, all of the mice were subjected to handling procedures (holding and weighing) from the first days of the experiment (experimental day 1). Anesthesia procedures with Me/Mi/Bu or isoflurane were performed on experimental day 8.

The following four groups were established as experimental groups (five animals per experimental group were used): (*i*) a control group that received only the handling treatment without restraint or anesthesia as controls for the entire experiment, (*ii*) an unanesthetized-restrained group that was subjected to restraint stress for 1 hr without anesthesia, (*iii)* an isoflurane group in which the mice were subjected to restraint stress for 1 hr under isoflurane anesthesia, and (*iv*) a Me/Mi/Bu group in which the mice were subjected to restraint stress for 1 hr under Me/Mi/Bu anesthesia. Restraint stress was applied by placing the mouse in a Decapicone® Disposable Mouse Restrainer (Braintree Scientific, Braintree, MA, USA), which is a disposable restraint garment for mice.

The following pharmaceutical grade products were used for Me/Mi/Bu: medetomidine hydrochloride (Dormitor®; Nippon Zenyaku Kogyo Co., Fukushima, Japan), midazolam hydrochloride (Dormicum®; Astellas Pharma, Tokyo, Japan), and butorphanol tartrate (Vetorphan®; Meiji Seika Pharma, Tokyo, Japan). The concentrations of medetomidine hydrochloride, midazolam hydrochloride, and butorphanol tartrate were adjusted to 0.75, 4.0, and 5.0 mg/kg, respectively. The Me/Mi/Bu mixture was administered intraperitoneally in all animals. Isoflurane (Viatris Pharma, Tokyo, Japan) was vaporized using an animal inhalation anesthesia device (model TK-6, Bio Machinery, Tokyo, Japan) and applied as inhalation anesthesia (concentration 2%, flow rate 1.5 L/min).

All mice were anesthetized with isoflurane and maintained under anesthesia, and after confirmation that there was no pain response, perfusion fixation was performed by the transcardiac method. The reagents and perfusion method used for perfusion fixation were as we have described [24, 25]. The perfused brain specimens were cut into serial frozen sections (frontal sections, 40-µm thickness per section) using a freezing microtome (Yamato, Osaka, Japan) for the regions corresponding to Figures 27–45 of The Mouse Brain Map by Franklin & Paxinos [4].

An immunohistochemical examination of the brain sections was performed by the floating method. The primary antibody used was anti-cFos antibody (ABE457, Lot# 3221531; Millipore, Westboro, MA, USA). The secondary antibody was anti-rabbit IgG (Elite Kit, Vector Labs, Burlingame, CA, USA), and staining was performed using the Vectastain ABC Kit and the ABC method [24, 25]. The specimens subjected to immunohistochemistry were observed under an optical microscope (DM1000; Leica, Wetzlar, Germany), and the sections corresponding to Figures 37–39 of The Mouse Brain Map [4] were selected at equality intervals (80 µm apart) for counting of the number of cFos-IR-positive cells in three consecutive sections (the distance between the anterior-to-posterior in PVN regions in three sections is approximately 240 µm). The PVN region in the selected section was photographed with a digital camera (DFC450C; Leica). The number of cFos-IR-positive cells in the PVN in each digital camera image was manually counted with the use of a colony counter pen (model #3133; As One, Tokyo, Japan), and the total number of cells counted from three consecutive sections on the same side was used as the cFos-IR value for each mouse. The cFos-IR-positive cells were measured in the magnocellular part of the PVN (PVN-M) areas (see Fig. 1).

To test for significant differences between experimental groups, we performed a one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test ($P<0.05$) using GraphPad Prism 7 (GraphPad Software, Boston, MA, USA) installed on a MacOS-based computer.

The cFos-IR expression results in the PVN are depicted in Figs. 1 and 2. The number of cFos-IR-immunopositive cells in the unanesthetized-restrained group was significantly higher than that in the control group subjected only to handling. This suggests that the study's restraint procedure induced a considerable degree of stress in the mice. In contrast, the expression of cFos-IR in the two anesthesia-restraint groups (the isoflurane group and Me/Mi/Bu group) exhibited a slight increase relative to the control group but a significant decrease compared to the no-anesthesia restrained group. These results confirm that anesthesia mitigates the physical stress induced by restraint at the central nervous system (brain) level, and that this inhibitory effect can be obtained with both isoflurane and Me/Mi/Bu.

When an animal is subjected to stress, the hypothalamic-pituitary-adrenal (HPA) axis is activated in response, resulting in the production of corticotropin-releasing hormone in the PVN of the hypothalamus. This in turn results in the secretion of glucocorticoids from the adrenal cortex via the adrenocorticotropic hormone (ACTH) of the pituitary gland [6, 12, 21]. It is well established that an activation of the production of stress-induced glucocorticoids can be evaluated by an increase in cFos in the mammalian PVN. Indeed, numerous stress investigations have used an increase in the number of cFos-IR cells in the PVN as a stress marker [11, 12, 16, 20]. Accordingly, we speculate that the increase in cFos-IR cells in the PVN observed in the present unanesthetized-restrained group (i.e., the increase in the PVN's neural activity) reflects the physical stress caused by the restraint treatment and the psychological stress caused by pain.

Conversely, the markedly reduced number of cFos-IR cells in the anesthesia-restraint groups (the Me/Mi/Bu and isoflurane groups) compared to the no-anesthesia restrained group demonstrates that both of these types of anesthesia effectively mitigate the physical and psychological stress associated with the restraint procedure. Moreover, although there was no significant difference in the number of cFos-IR cells between the Me/Mi/Bu and isoflurane groups, the Me/Mi/Bu group tended to have a lower number of cFos-IR cells than the isoflurane group. This suggests that the anesthetic effect of Me/Mi/Bu is equivalent to, or even more effective than, that of isoflurane in reducing stress.

An earlier investigation evaluated the effects of various anesthetics on rats by comparing the low induction of cFos-positive cells in the PVN [15]. It has been reported that urethane, which is thought to have minimal analgesic efficacy, exhibits a high level of cFos-IR

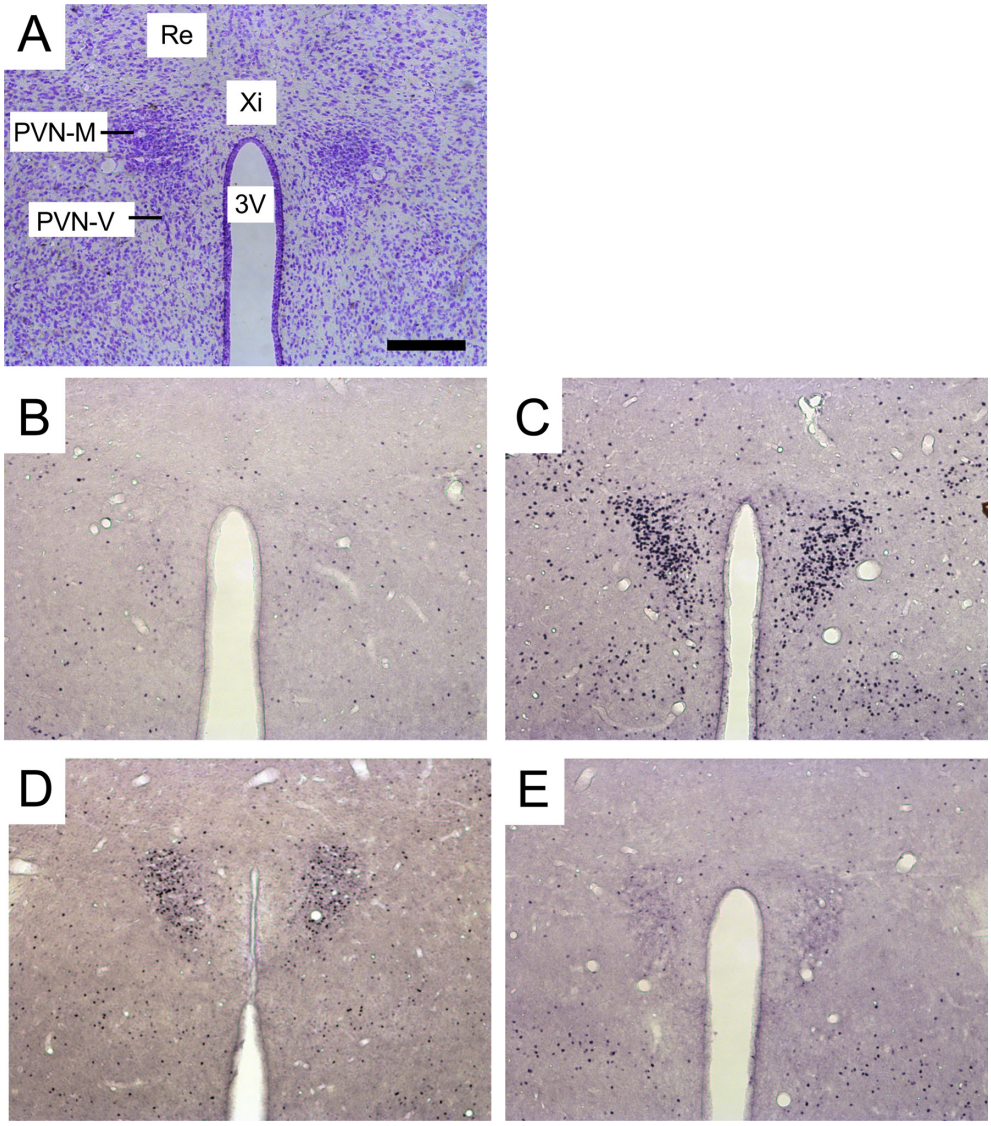

Fig. 1. Representative examples of cFos immunohistochemistry of brains from individual mice under different restraint and anesthesia conditions. The images compare the expression of cFos-immunoreactivity (IR) in the paraventricular nucleus of the hypothalamus (PVN), which is associated with stress sensitivity. **A:** Representative PVN location used for in cFos-IR analysis (Nissl staining; adjacent section to B). **B:** Control. **C:** Restrained under no anesthesia, **D:** Restrained under isoflurane anesthesia. **E:** Restrained under the combination of medetomidine, midazolam, and butorphanol (Me/Mi/Bu) anesthesia. The cFos-IR were observed mainly in the magnocellular part of the PVN (PVN-V), which is closely related to stress. PVN-M: magnocellular part of the PVN. PVN-V: ventral part of the PVN. Re: renuiens thalamic nucleus. Xi: xiphoid thalamic nucleus. 3V: 3rd ventricle. Scale bar in A: 200 µm.

expression in the PVN. Conversely, anesthetics with recognized analgesic properties such as halothane, sodium pentobarbital, and a combination of fentanyl with midazolam, display a low level of cFos-IR expression [15]. In a study examining the anesthetic effects of sodium pentobarbital—which is still used as a euthanasia agent in veterinary medicine and laboratory animals in Europe and the U.S. and until recently was also widely used as a surgical anesthetic for small laboratory animals—and using the expression of cFos protein in the brain as an indicator, it was observed that upon the awakening of mice from pentobarbital-anesthesia, the increase in neural activity was very much more pronounced in the PVN than in other neuronal nuclei [22]. Moreover, when the anesthetic effect of isoflurane on mice was observed in conjunction with PVN neural activity, it was noted that the expression of cFos decreased with the induction of anesthesia and increased in synchronization with the animals' awakening from anesthesia [23]. In other words, the decrease and activation of PVN neural activity appears to be synchronized with the state of anesthesia induced by surgical anesthetics. It has been reported that there is no sex difference in c-Fos expression in the PVN of mice in response to acute restraint stress [2]. However, in the PVN under non-stressed conditions, the proportion of neurons co-expressing corticotropin-releasing hormone (CRH) and estrogen receptor α is significantly higher in males [14]. In the present study, only male mice were used, but the stress-relieving effects of anesthesia in the central nervous system may exhibit sex differences. Therefore, future studies will require female mice in addition to male data.

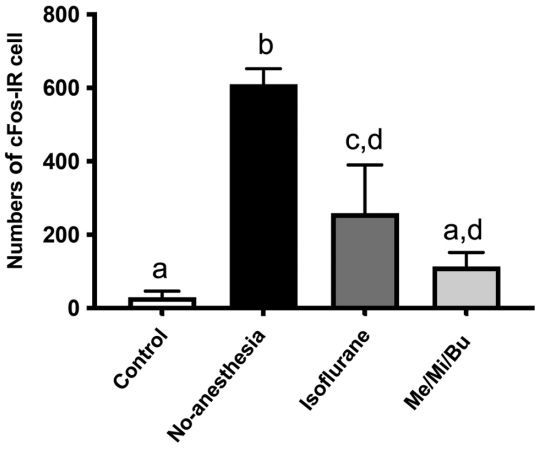

Fig. 2. The quantification of cFos-IR cell counts in the paraventricular nucleus of the hypothalamus showed that the isoflurane and the combination of medetomidine, midazolam, and butorphanol (Me/Mi/Bu) anesthesia treatment groups had significantly lower numbers of cFos-IR cells compared to the no-anesthesia restraint group. Control: only the handling treatment without restraint or anesthesia. No-anesthesia: restraint stress for 1 hr without anesthesia. Isoflurane: restraint stress for 1 hr under isoflurane anesthesia. Me/Mi/Bu: restraint stress for 1 hr under Me/Mi/Bu anesthesia. Five mice per group were examined. Differing lowercase letters indicate significant differences ($P<0.05$).

Considering our present findings in conjunction with the above-cited research describing PVN neural activity in relation to the effects of various anesthetics, we suggest that Me/Mi/Bu has the same effect on the brain as isoflurane as a surgical anesthetic.

Further cFos immunohistochemical analyses of brain regions that are involved in anesthesia other than the PVN and a comparison of the EEG pattern of Me/Mi/Bu-treated animals with that of isoflurane-treated animals, which that are undergoing actual surgical procedures, can be expected to demonstrate whether Me/Mi/Bu acts as a surgical intoxicant.

POTENTIAL CONFLICTS OF INTEREST. The authors report no conflicts of interest in the conduct of this work.

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