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Effect of combining anesthetics in neonates on long-term cognitive function

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ABSTRACT

Background: With growing evidence that anesthesia exposure in infancy affects cognitive development, it is important to understand how distinct anesthetic agents and combinations can alter long-term memory. Investigations of neuronal death suggest that combining anesthetic agents increases the extent of neuronal injury. However, it is unclear how the use of simultaneously combined anesthetics affects cognitive outcome relative to the use of a single agent.
Methods: Postnatal day 7 (P7) male rats were administered either sevoflurane as a single agent or the combined delivery of sevoflurane with nitrous oxide at 1 Minimum Alveolar Concentration for 4 h. Behavior was assessed in adulthood using the forced alternating T-maze, social recognition, and context-specific object recognition tasks.
Results: Animals exposed to either anesthetic were unimpaired in the forced alternating T-maze test and had intact social recognition. Subjects treated with the combined anesthetic displayed a deficit, however, in the object recognition task, while those treated with sevoflurane alone were unaffected.
Conclusion: A combined sevoflurane and nitrous oxide anesthetic led to a distinct behavioral outcome compared with sevoflurane alone, suggesting that the simultaneous use of multiple agents may uniquely influence early neural and cognitive development and potentially impacts associative memory.
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1. Introduction

Every day, children around the world undergo general anesthesia for various procedures and operations. Epidemiologic studies have raised concerns that humans are susceptible to long-term effects on learning and memory following exposure to anesthesia at an early age (Flick et al., 2011; Wilder et al., 2009). Animal models reveal that neonates exposed to anesthetics suffer extensive neuronal death and persistent memory deficits (Jevtovic-Todorovic et al., 2003, 2012; Nikizad et al., 2007; Istaphanous et al., 2011; Stratmann et al., 2009a; Shih et al., 2012; Ramage et al., 2013; Boscolo et al., 2012; Rizzi et al., 2010; Gentry et al., 2013). With increasing evidence regarding the detrimental effects of neonatal anesthesia exposure, it is important to understand how anesthetic agents and combinations of agents might influence cognitive development.

Sevoflurane is a volatile anesthetic frequently used in children as a sole agent or in conjunction with nitrous oxide, a separate type of anesthetic. These anesthetics exert their effects via different mechanisms – sevoflurane is believed to be a gamma-aminobutyric acid ($GABA_A$) agonist (Franks and Lieb, 1994; Ishizeki et al., 2008) while nitrous oxide acts as an *N*-methyl-D-aspartate (NMDA) receptor antagonist (Sanders et al., 2008; Jevtovic-Todorovic et al., 1998; Nagele et al., 2004). In the past, studies of neuronal death have indicated that the co-administration of a GABA agonist with an NMDA antagonist might result in greater neuronal death than either agent

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individually (Fredriksson et al., 2007). In addition, others have shown that the addition of nitrous oxide to another volatile anesthetic leads to increased neurotoxicity (Ma et al., 2007; Zhen et al., 2009).

Although there are numerous studies of cell death (Istaphanous et al., 2011; Rizzi et al., 2010; Fredriksson et al., 2007; Ma et al., 2007; Zhen et al., 2009; Creeley et al., 2014; Deng et al., 2014), there is a lack of behavioral experiments to accompany them. The important outcome of cognition and memory after anesthetic exposure is, therefore, understudied. As a result, it remains unclear whether anesthetics may induce different long-term effects on memory when used in combination rather than as individual agents.

Minimum Alveolar Concentration (MAC) is the minimum amount of inhaled anesthetic required to prevent movement in response to a painful stimulus and is a reliable measure of potency (Eger et al., 1965; Merkel and Eger, 1963). Unlike in adult rodents, MAC in newborns is not a fixed concentration but decreases over time and involves continual adjustment of the concentration (Stratmann et al., 2009a; Kodama et al., 2011). By adjusting to 1 MAC, we are able to compare cognitive outcomes from anesthetics that are similar in potency (Ramage et al., 2013). In the present study, we investigated whether a combined anesthetic of sevoflurane and nitrous oxide would lead to a different behavioral outcome than sevoflurane alone. Following exposure to 1 MAC of either anesthetic as newborns, long-term memory was evaluated by testing subjects in the forced alternating T-maze, social recognition, and an object recognition task relying on associative learning.

2. Materials and methods

2.1. Subjects

All experiments were conducted with approval from the Institutional Animal Care and Use Committee at University of California, San Francisco. Dams with postnatal-day 6 (P6) male Sprague-Dawley pups were purchased from Charles River Laboratories (Glilroy, CA). At P7, pups were randomized into three groups – control ($n = 29$), anesthesia with sevoflurane ($n = 54$), anesthesia with sevoflurane and nitrous oxide ($n = 27$). Following anesthesia, animals were cross-fostered among the dams until weaning at P21. They were then kept in standard lab housing with 12-h light–dark cycle and given ad libitum access to food and water prior to cognitive testing. During testing, animals were housed individually and food restricted as described for each experiment below.

2.2. Anesthesia

Anesthetic delivery was performed similarly to what we have reported before (Ramage et al., 2013; Stratmann et al., 2009b). Briefly, treatment animals received either sevoflurane as a single agent or the simultaneously combined treatment of sevoflurane with nitrous oxide for a total of 4 h. Each anesthetic regimen was adjusted to 1 Minimum Alveolar Concentration (Eger et al., 1965). Sevoflurane was administered in air and oxygen (FiO_2 25%), and MAC was determined by tail clamping every 15 min and anesthetic concentration was adjusted so that 50% of animals would respond to the stimulus (Ramage et al., 2013; Stratmann et al., 2009b). In the combined treatment, nitrous oxide was held constant at 70% while sevoflurane concentration was adjusted to achieve 50% movement in response to tail clamping. Control rats were treated in an identical manner for 4 h without being exposed to anesthetic. Animals were kept on a warming blanket in the chamber and temperatures were measured with an infrared laser thermometer and maintained with a goal of 35 °C, the average skin temperature of non-anesthetized control pups in a huddle without the dam.

2.3. Forced alternating T-maze

2.3.1. Apparatus

Testing was conducted in a T-maze apparatus made of wood with a detachable stem (length 55 cm, width 10 cm) and crosspiece (length 91 cm, width 10 cm). Food wells (diameter 2.5 cm, depth 2 cm) at the ends of each arm were recessed into the maze track so they were not visible from the stem. The food wells contained a full size reward (Silly Circles, Safeway Kitchens) fixed between two cup-shaped filters so both baited and unbaited arms had the same smell. Clear acrylic was used for the maze walls with guillotine-style doors (width 10 cm, height 20 cm) at the maze arms and start area. Testing occurred in a 3-m square area enclosed in black felt curtain with visual reference cues on each wall.

2.3.2. Habituation and testing

Subjects from the control group ($n = 29$), sevoflurane group ($n = 41$), and sevoflurane with nitrous oxide group ($n = 26$) began behavioral testing on P69. From P69 to P85, subjects were food restricted and weighed daily to achieve 85–90% normal bodyweight. At P69, animals were given two 5-min trials of free exploration in the open T-maze with rewards placed in both food wells and along the floor throughout the maze. Animals not moving after 5 min were guided down the stem and given an additional minute for exploration. At P70–74, habituation continued without guided exploration or rewards along the track.

Subjects began forced-alternation testing in the T-maze at P76 between 0700 and 1900 h. Testing occurred over a period of 10 days with 6 trials per day. Each trial consisted of two runs – an "information" run and a "choice" run. During the information run, one of the two arms was closed so the rat would have only one option (left or right) in order to obtain the reward. In the subsequent choice run, both arms were open, and only the opposite arm as the previous run contained the reward. If the animal entered the same arm it had already visited, then it was negatively reinforced by being confined for 10 s within the arm lacking the reward. The direction of the choice and information runs were randomized for each trial using a computer so that every animal was given an equal number of left and right entries but the order was variable. Subjects were introduced into the maze facing away from the crosspiece during each run and given 3 min to commit to an arm. Commitment to an arm was established when a subject's hind legs entered the arm. Any animal unable to commit to an arm within 3 min was returned to its cage without a reward. Trials in which the rat did not make a choice were not scored and only sessions during which an animal made a choice in at least 4 out of six trials were used in the final results. The maze was wiped clean between subjects using 70% ethanol and the same handlers were used throughout all behavioral experiments.

On days 1–8, the delay between the information and choice run was 5 s. During days 9 and 10, animals underwent delayed forced alternation testing with a 30-s delay between the information run and choice run (based on validation testing, animals had fewer correct choices but still performed the task at 30 s). After completing the information run, animals were placed in the closed start area and confined for 30 s before opening the door to begin the choice run.

2.4. Social interaction

Upon completion of the T-maze test, rats were given unrestricted access to food and water. To assess social behavior, subjects were presented simultaneously with a female rat and novel object and assessed whether they spent more time investigating the social target. Six adult female Sprague Dawley rats were used as social targets and housed individually prior to testing. They were introduced within cages or "holders" and placed in the arena opposite the novel object. The male subject was given 5 min for exploration while interactions were recorded with a video camera (SONY HDR-CX190) mounted 2 m above the box.

Investigation of the female was defined as any direct contact with the nose or paws, as well as sniffing toward any part of the female including the tail if it extended outside of the holder. Not included was time spent sniffing toward the empty top portion of the holder or circling without pausing to sniff. Investigation of the object was defined as sniffing or placing the nose within 1 cm of and oriented toward the object. This excluded merely using the object as a support during rearing. Observers blind to group assignment were used to record the investigation times.

2.5. Social recognition

Subjects were separately tested in social recognition using a two-trial discrimination model. Exposure to a single female was followed by a second exposure to the same (familiar) female and a novel female. Testing occurred during the light cycle, between 0900 and 1700 h, and each rat was tested in its home cage (20 cm × 23 cm × 46 cm). The same adult females were used as social targets, although testing occurred more than 1 week after the social interaction task so subjects would not recall the stimulus animals.

In the first exposure, the male subject was given 5 min to investigate a single female. After a 60-min delay, the first female was presented simultaneously with a novel female. The male subject was then given 3 min to explore the two female rats. The test phase was recorded and investigation times were later scored by an observer blind to group assignment.

2.6. Object-place-context recognition task

2.6.1. Contexts

For object recognition, two testing arenas, hereafter referred to as "contexts," were used that were distinct in texture and appearance. Each context had 61 cm square base and 30 cm high walls. Context 1 had a base covered with white PEVA shower liner and walls covered with brown cardboard. Context 2 had base and walls made of black plastic. Each context had different visual cues on three walls. All subjects were habituated to each context prior to testing.

2.6.2. Testing

Subjects were tested in context-specific object recognition, which took place between 0700 and 1900 h using the arrangements shown in Fig. 1. In the first