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Effects of isoflurane and ethanol administration on c-Fos immunoreactivity in mice

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Abstract

Noninvasive functional imaging holds great promise for the future of translational research, due to the ability to directly compare between preclinical and clinical models of psychiatric disorders. Despite this potential, concerns have been raised regarding the necessity to anesthetize rodent and monkey subjects during these procedures, because anesthetics may alter neuronal activity. For example, in studies of drugs of abuse and alcohol, it is not clear to what extent anesthesia can interfere with drug-induced neural activity. Therefore, the current study investigated whole brain c-Fos activation following isoflurane anesthesia as well as ethanol-induced activation of c-Fos in anesthetized mice. In the first experiment, we examined effects of 1 or 3 sessions of gaseous isoflurane on c-Fos activation across the brain in male C57BL/6J mice. Isoflurane administration led to c-Fos activation in several areas, including the piriform cortex and lateral septum. Lower or similar levels of activation in these areas were detected after three sessions of isoflurane, suggesting that multiple exposures may eliminate some of the enhanced neuronal activation caused by acute isoflurane. In the second experiment, we investigated the ability of ethanol injection (1.5 or 2.5 g/kg i.p.) to induce c-Fos activation under anesthesia. Following three sessions of isoflurane, 1.5 g/kg of ethanol induced c-Fos in the central nucleus of amygdala, piriform cortex, and centrally-projecting Edinger-Westphal nucleus. This induction was lower after 2.5 g/kg of ethanol. These results demonstrate that ethanol-induced neural activation can be detected in the presence of isoflurane anesthesia. They also suggest, that while habituation to isoflurane helps reduce neuronal activation, interaction between effects of anesthesia and alcohol can occur. Studies using fMRI imaging could benefit from using habituated animals and dose response analyses.

Keywords

Isoflurane; c-Fos; Alcohol; Anesthesia

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1.1 Introduction

For several decades, neuroanatomical techniques for measuring neuronal activation have been utilized to understand the neurobiological consequences of administration of drugs of abuse. One such technique utilizes postmortem measurement of immediate early genes (IEGs), which are expressed following neuronal activation and lead to IEG-encoded transcription factors capable of affecting gene expression. One such IEG, c-Fos, is expressed at very low levels basally, but is rapidly and transiently induced following a variety of stimuli including administration of drugs of abuse (Graybiel et al., 1990; Ryabinin et al., 2000). These types of studies have created a foundation for understanding the neuroanatomical and neurobiological mechanisms of drug abuse, but are restricted to use within preclinical models.

On the other hand, non-invasive neuroimaging techniques are available for use in human and animal models. With the advent of techniques like functional magnetic resonance imaging (fMRI), it is now possible to conduct translational studies focused on identical measurements of brain activity in preclinical and clinical models. Functional MRI measures neural activity via changes in oxygen metabolism and has been extensively characterized in humans. More recently, such characterization has been taking place in animal models, including rodents and monkeys (Miranda-Dominguez et al., 2014; Stafford et al., 2014). These studies often require that the subjects be placed under prolonged anesthesia during imaging to control for behavioral stress and movement. However, it is unclear how anesthesia might interfere with drug induced neuronal activation in imaging studies, and this represents an experimental confound that could manifest in several different ways. First, it is possible that the anesthetic and drug of interest act on overlapping neurotransmitter systems or neural circuitry. For example, both alcohol and typical injectable anesthetics (eg: barbiturates) have dose-dependent actions at the GABA_A receptor (Olsen et al., 2007; Santhakumar et al., 2007; Kotani and Akaike, 2013), therefore, the effects of alcohol in awake subjects or those under anesthesia may differ. In fact, it has been demonstrated that actions of alcohol on c-Fos are absent during pentobarbital anesthesia (Ryabinin, 2000). Relatedly, another possible confound is that certain neuronal effects of drugs of abuse depend upon perception, and these effects are absent when the subject is anesthetized. Conversely, many pharmacological effects of drugs of abuse remain, which may represent a benefit provided by investigating neural activity under anesthesia (Torres and Rivier, 1994; Ryabinin, 2000). Finally, the initial moments of anesthesia induction could cause novelty- or stress-induced changes in neural activity that could interfere with detection of the pharmacological action of the drug. It has been thoroughly demonstrated that although c-Fos induction is sensitive to novelty/stress, this is an effect that habituates following repeated exposure (Melia et al., 1994; Radulovic et al., 1998; Ryabinin et al., 1999). Therefore, habituation to repeated anesthesia could eliminate this concern. Considering this, the current studies were designed to first investigate c-Fos expression following single or repeated isoflurane exposures, and then test alcohol-induced c-Fos activation under isoflurane anesthesia. Accordingly, we hypothesized that multiple isoflurane exposures would induce less overall c-Fos activation, and would be less likely to interfere with alcohol induced c-Fos expression.

2.2 Experimental Procedures

2.2.1 Animals

Adult male C57BL/6J mice were used in all experiments (n = 4-8/group). Mice were delivered from The Jackson Laboratory (Sacramento, CA) at 7-8 weeks of age, housed 3-5 per cage, and spent at least 1 week acclimating to our colony room prior to experiments (12:12 schedule; lights on 06:00 hours). For all experiments, mice were housed in our animal colony, which is a temperature- and humidity-controlled environment with *ad libitum* access to food (LabDiet 5001; LabDiet, Richmond, IN) and tap water. Following the termination of experiments, all mice were sacrificed via cervical dislocation. All protocols were approved by the Oregon Health & Science University animal care and use committee and performed within the National Institutes for Health Guidelines for the Care and Use of Laboratory Animals, as well as the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research.

2.2.2 Drugs

Ethanol (EtOH) solutions for injection (20% v/v; 1.5g/kg or 2.5g/kg, i.p.) were prepared in saline from 95% ethyl alcohol. Isoflurane (Iso) 1-4% was delivered in oxygen via a precision vaporizer (DatexOhmeda, WI).

2.2.3 Experimental Procedure

All experiments occurred between 8:00am-1:00pm. Animals were assigned to one of six treatment groups: Habituation Only (Hab), Isoflurane 1x (Iso/1), Isoflurane 3x (Iso/3), Isoflurane + Saline (Iso/Sal), Isoflurane + one of two ethanol doses EtOH (1.5g/kg; Iso/EtOH1.5 or 2.5g/kg; Iso/EtOH2.5). *See* Figures 1A & 2A for timelines.

Habituation: All mice were subjected to a habituation procedure, which consisted of transport to the experimental room on 2 consecutive days. Mice remained in their homecages and were allowed to acclimate to the experimental room for 135 min before transport back to the animal colony (Fig 1A, 2A).

All treatment groups remained in the experimental room for a total of 135 minutes. Following habituation all groups were transported to the experimental room and allowed 15 mins of acclimation before group treatments were given. For the Hab mice, the 15 minutes of acclimation was followed by an additional 2 hours in the experimental room prior to sacrifice. All other groups were subjected to Iso treatment following the 15 min of acclimation to the room.”. During Iso treatment, animals were monitored for respiration changes and movement, to ensure maintenance of anesthesia. Iso treatment consisted of placement in the anesthesia chamber, induction via 4% Iso for five minutes and maintenance at 1% Iso for 120 mins. Following the final experimental treatment, all mice were sacrificed via cervical dislocation in an adjacent experimental room. Iso/1 mice received the Iso treatment regimen one time. Iso/3 mice were subjected to Iso treatment regimen on 3 consecutive days. For Iso/Sal and Iso/E groups, the Iso procedure was identical to the Iso/3 group, with the exception that 20 mins after placement in the induction chamber, mice were injected (i.p.) and placed back the chamber for the remaining hour. On the first two days all

mice received saline injections, on the third day mice received either saline, or their assigned EtOH dose (Fig 1A, 2A).

2.2.4 Tissue Processing

Immediately following cervical dislocation, brains were extracted and post-fixed for 24 h in 2% paraformaldehyde/phosphate buffered saline (PBS), followed by cryopreservation in 20% and then 30% sucrose/PBS. Coronal sections were taken at 30 μ m across the entire brain. Brain regions were defined by the Paxinos and Franklin (2008) Mouse Brain Atlas parameters. The tissue was processed for c-Fos immunohistochemistry using standard avidin-biotin-DAB protocols (Ryabinin et al., 2000; Bachtell et al., 2002). Immunopositive cells were counted manually using a Leica DM4000 microscope (Bartels and Stout, Inc., Bellevue, WA, USA) in slices collected across the whole brain. All counts were conducted by an experimenter that was blind to treatment condition. The c-Fos positive cell counts were averaged across 3-5 slices for each region. This average served as a single data point for statistical analysis.

2.2.5 Statistical Analyses

Due to unequal variances between groups, a Kruskal-Wallis one-way ANOVA was used to analyze the number of c-Fos positive cells per treatment group. Dunn's posthoc analysis was used when appropriate. All data are presented as mean and standard error of the mean.

3.1 Results

3.1.1 Isoflurane-induced c-Fos activation

Slices across the whole brain of individual animals were visually scanned to determine the location of enhanced c-Fos immunoreactivity. The majority of brain areas showed negligible c-Fos expression irrespective of group. However, any enhancement in c-Fos activation seen following one session of isoflurane was attenuated following three sessions. In fact, there were no cases in which three sessions led to significantly increased c-Fos activation compared to one session (Table 1). However, this trend reached statistical significance only in two brain areas. Thus, compared to habituation only, one session of isoflurane significantly enhanced c-Fos activation in the piriform cortex (PIR, $\chi^2(2) = 7.769$, $p = 0.011$; Table 1 & Fig 1B) and the dorsal lateral septum (LSD, $\chi^2(2) = 7.769$, $p = 0.01$; Table 1 & Fig 1C) that was no longer significant after three sessions according to Kruskal-Wallis and Dunn's posthoc analysis ($p < 0.05$). Furthermore, isoflurane enhanced c-Fos in the ventral lateral septum (LSV), although this effect reached statistical significance only after three sessions ($\chi^2(2) = 8.493$, $p < 0.001$; Dunn's $p < 0.05$; Fig 1D) when compared to Hab mice.

3.1.2 Alcohol induced c-Fos activation under isoflurane anesthesia

Due to the sparsity of isoflurane-induced c-Fos activation following 3 sessions, we decided to use this procedure to examine alcohol induced c-Fos activation under anesthesia (Fig 2A). Utilizing this timeline, we were able to see measureable enhancements in c-Fos in two brain areas. In fact, injection of 1.5 g/kg EtOH during the third isoflurane session led to significant c-Fos activation in the central nucleus of amygdala ($\chi^2(2) = 6.017$, $p = 0.043$; Table 1 & Fig 2B) and centrally-projecting Edinger-Westphal nucleus ($\chi^2(2) = 6.097$, $p = 0.041$; Table 1 &

Fig 2D) compared to saline injected mice, according to Kruskal-Wallis and Dunn's posthoc ($p < 0.05$). A higher dose of EtOH (2.5 g/kg) did not lead to a significant enhancement in c-Fos compared to saline treated mice ($p > 0.05$; Table 1 & Fig. 2B-D) in any of these areas.

4.1 Discussion

Preclinical imaging studies utilizing fMRI to investigate drugs of abuse are regularly conducted on anesthetized subjects. Utilizing an anesthesia procedure that is common for fMRI studies in rodents, we observed that isoflurane does not lead to robust c-Fos responses, supporting its applicability to such studies. The only area that maintained a high level of c-Fos immunoreactivity after three sessions of isoflurane was the LSV, suggesting that this area is involved in pharmacological effects of this anesthetic. To our knowledge, this brain area has never been investigated previously in studies on isoflurane. However, it sends strong projections to the medial septum (Risold and Swanson, 1997), an area well known to regulate general anesthesia (Leung et al., 2014). In contrast, PIR and LSD showed c-Fos induction following the first isoflurane exposure, which decreased after 3 exposures. PIR and LSD are known to respond when animals are exposed to environmental novelty and stress (Emmert and Herman, 1999; Menard et al., 2004; Stone et al., 2006). It is likely that these brain areas were activated due to the novelty and stress factors associated with the first experience of anesthesia. This suggests that habituation to anesthesia could result in less interference with neural activity elicited by the pharmacological treatment under study.

Accordingly, we demonstrate that following 3 sessions of isoflurane anesthesia, we are able to detect alcohol induced c-Fos activation in anesthetized mice, indicating that the low level of isoflurane-induced activation did not interfere with the ability of alcohol to induce c-Fos activation. Interestingly, this occurred following administration of the lower (1.5 g/kg) but not higher dose of EtOH (2.5 g/kg). This reduction in c-Fos suggests that there may be pharmacological interactions between isoflurane anesthesia and higher doses of alcohol. It is possible that this effect could be explained by actions on glutamate, as both alcohol and isoflurane have been shown to dose dependently inhibit this system (Carboni et al., 1993; Sandstrom, 2004; Möykkynen and Korpi, 2012). However, more in depth neurobiological and electrophysiological studies would need to be conducted to specifically address this possibility. Additionally, our findings agree with recent studies which demonstrated that cocaine-induced Fos expression is measureable under isoflurane but not α -chloralose anesthesia (Kufahl et al., 2009; 2015) suggesting that isoflurane is a good candidate for these types of experiments. Nonetheless, it is clear that thorough dose response studies need to be conducted examining interactions between specific drugs of abuse and anesthetics prior to functional imaging studies.

In the current studies, we used the IEG c-Fos as a measure of neuronal activation, as it is the most widely used functional neuroanatomical marker of activated neurons, and due to the fact that previous efforts from our laboratory and others have extensively mapped ethanol-induced c-Fos activation (Ryabinin et al., 2000; Bachtell et al., 2002; Sharpe et al., 2005). In fact, these studies demonstrated that the centrally projecting Edinger-Westphal nucleus (EWcp) is the only area that consistently contains enhanced c-Fos following alcohol administration by an experimenter (Chang et al., 1995; Ryabinin, 1998; Bachtell et al.,

2002) and following alcohol self-administration (Topple et al., 1998; Bachtell et al., 1999; Weitemier et al., 2001; Ryabinin et al., 2003; Sharpe et al., 2005). The central nucleus of amygdala (CeA) was observed to be consistently activated following injections of alcohol in awake animals (Chang et al., 1995; Hitzemann and Hitzemann, 1997; Ryabinin et al., 1997) and much less consistently following alcohol self-administration (Bachtell et al., 1999; Ryabinin et al., 2000). In the current studies, the induction of c-Fos in CeA and EWcp demonstrate that the effect of alcohol on c-Fos activation is predictable under anesthesia, when utilizing habituation and a proper dose response. Moreover, this finding indicates that c-Fos in these brain regions is due to pharmacological effects of alcohol, and not due to perception of cues related to alcohol intoxication. This notion is in agreement with studies showing importance of CeA and EWcp for regulation of alcohol consumption (Hyytia and Koob, 1995; Ryabinin and Weitemier, 2006; Gilpin et al., 2015). Remarkably, CeA and EWcp are also the only brain regions in which lesions have been shown to selectively decrease alcohol preference and intake in mice (Bachtell et al., 2004; Dhaher et al., 2008).

Our studies utilized c-Fos as a marker of neural activity, yet much could be learned from other indicators of neuronal activity, such as ETS-related gene 1 (Erg1) or Activity-regulated cytoskeletal-associated protein (Arc). In the future it will be essential to examine additional markers of neuronal activity, as well as alternate anesthetics to fully characterize anesthesia-induced neuronal activation. In addition, it would be valuable to perform similar studies during the dark (active) portion of the circadian cycle, as most human studies are performed during daytime.

We chose to investigate isoflurane anesthesia, because it is commonly used in imaging studies due to its ability to provide a stable condition, allow for easy control of anesthesia depth, and be used for survival studies (Sakai et al., 2005). However, other anesthetics such as medetomidine and α -chloralose are often used in functional imaging studies and should be investigated in more detail. Finally, it will be essential to fully elucidate how IEG neuroanatomical markers compare to activation of the BOLD signal in functional imaging studies. In depth investigation of the relationship between functional (eg: BOLD signal) and neuroanatomical (IEG's) markers of brain activation will further the neurobiological understanding of these processes and enhance their translational capacity. In sum, the results of this study indicate that isoflurane is a good candidate for anesthesia during functional imaging studies in the mouse. Specifically, isoflurane anesthesia is appropriate for examining drug-induced neuronal activation, especially following sufficient habituation to the anesthesia process.

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Highlights

- One session of isoflurane anesthesia induced c-Fos in piriform cortex and lateral septum.
- Isoflurane-induced c-Fos habituated following repeated anesthesia in all areas except LSV.
- Ethanol-induced c-Fos is detected under isoflurane anesthesia in EWcp and CeA.

Figure 1.

Effects of Isoflurane on c-Fos expression (n= 4-7/group). A) Experimental timeline: Acclimation (15 or 120 minutes; grey bars), isoflurane sessions (1-4%; gradient bars) and sacrifice (arrows; SAC). Groups: Habituation only (Hab, n= 4), 1 session of Isoflurane (Iso/1, n= 4) and 3 sessions of Isoflurane (Iso/3, n=7). B) Representative photomicrograph of c-Fos IR in the piriform cortex (PIR); Bregma 0.74mm. C) c-Fos expression in the PIR. D) c-Fos expression in the dorsal lateral septum (LSD). E) c-Fos expression in the ventral lateral septum (LSV). Significant differences according to Kruskal Wallis one way ANOVA followed by Dunn's posthoc analyses: * $p < 0.05$; ** $p < 0.001$.

Figure 2.

Effects of ethanol on c-Fos expression in anesthetized mice ($n = 5-8$ /group). A) Experimental timeline: Acclimation (15 or 120 minutes; grey bars), isoflurane sessions (1-4%; gradient bars), injections (Sal, or EtOH; syringe) and sacrifice (arrows; SAC). Groups: 3 sessions isoflurane with saline injections only (Iso/S, $n = 5$), or 3 sessions of isoflurane with an EtOH injection on the final day (Iso/E, $n = 7-8$). B) Representative photomicrograph of c-Fos IR in the centrally-projecting Edinger-Westphal nucleus (EWcp). C) Expression of c-Fos in the central nucleus of amygdala (CeA). D) Expression of c-Fos in CeA. Significant differences according to Kruskal Wallis one way ANOVA followed by Dunn's posthoc analyses: $*p < 0.05$.

Table 1

Number of c-Fos-positive cells (mean $\pm$ standard error) across brain regions (defined by Paxinos & Franklin, 2008).

Brain area	Hab	Iso/1	Iso/3	Kruskal Wallis 2(2)	Iso/Sal	Iso/EtOH1.5	Iso/EtOH2.5	Kruskal Wallis 2(2)
Nucleus Accumbens Shell	0.17 $\pm$ 0.1	0.33 $\pm$ 0.24	0.0 $\pm$ 0.0	3.86	0.59 $\pm$ 0.25	0.6 $\pm$ 0.29	0.39 $\pm$ 0.20	0.47
Nucleus Accumbens Core	0	0	0	-	0.31 $\pm$ 0.12	0.2 $\pm$ 0.2	0.39 $\pm$ 0.25	0.56
Central Nucleus of Amygdala	0	0	0	-	0.71 $\pm$ 0.24	1.77 $\pm$ 0.32 *	1.58 $\pm$ 0.8	6.02#
Piriform Cortex	0.5 $\pm$ 0.35	24.69 $\pm$ 7.98 *	3.63 $\pm$ 1.75	7.77#	0	0	0	-
Dorsal Lateral Septum	0.06 $\pm$ 0.06	3.06 $\pm$ 0.8 *	1.71 $\pm$ 0.77	7.84**	5.06 $\pm$ 1.8	13.5 $\pm$ 2.79	8.1 $\pm$ 1.65	5.7
Ventral Lateral Septum	0.08 $\pm$ 0.08	7.92 $\pm$ 1.04	8.72 $\pm$ 1.39 **	8.49**	10 $\pm$ 2.98	14 $\pm$ 3.97	13.9 $\pm$ 2.16	2.98
Infralimbic Cortex	0	0	0	-	0.69 $\pm$ 0.42	0.8 $\pm$ 0.37	0.43 $\pm$ 0.3	1.01
Paraventricular Thalamic Nucleus	2.38 $\pm$ 1.16	8.5 $\pm$ 2.55	8.25 $\pm$ 2.75	3.16	2.63 $\pm$ 1.47	0.4 $\pm$ 0.4	2.14 $\pm$ 0.81	2.51
Dentate Gyrus	6 $\pm$ 0.94	5.25 $\pm$ 2.47	3.08 $\pm$ 1.43	1.94	0.94 $\pm$ 0.8	1.8 $\pm$ 1.8	0.5 $\pm$ 0.24	0.34
Dorsal Medial Hypothalamus	0.25 $\pm$ 0.25	5.25 $\pm$ 3.31	2.25 $\pm$ 1.08	2.67	1.31 $\pm$ 0.92	0.4 $\pm$ 0.4	0.57 $\pm$ 0.39	0.18
Posterolateral Cortical Amygdala	1 $\pm$ 0.71	1.88 $\pm$ 0.88	0.25 $\pm$ 0.11	2.72	0.13 $\pm$ 0.13	0 $\pm$ 0	0.57 $\pm$ 0.57	0.74
Posteromedial Cortical Amygdala	0.75 $\pm$ 0.43	1.75 $\pm$ 1.27	0.17 $\pm$ 0.17	3.1	0.12	0	0	1.5
Periaqueductal Gray	1.75 $\pm$ 1.44	1.63 $\pm$ 1.31	3.67 $\pm$ 2.12	1.15	1.5 $\pm$ 0.52	4.4 $\pm$ 1.36	1.36 $\pm$ 0.40	3.65
Ventral Tegmental Area	0.75 $\pm$ 0.75	0	0	-	0	0	0	-
Substantia Nigra	0.13 $\pm$ 0.13	0.13 $\pm$ 0.13	0	-	0	0	0	1.63
Centrally Projecting Edinger-Westphal	0.6 $\pm$ 0.28	0.79 $\pm$ 0.63	1.12 $\pm$ 0.54	2.08	3.11 $\pm$ 0.85	11.1 $\pm$ 2.9 *	5.98 $\pm$ 2	6.1#

Bold font represents significant differences based upon Kruskal-Wallis one way ANOVA ([#]) at $p < 0.05$. Specific groups differences based upon Dunn's posthoc analyses:

* $p < 0.05$

** $p < 0.001$.