Neuroscience in Anesthesiology and Perioperative Medicine - Isoflurane inhibits synaptic calcium reuptake in mouse neurons in an ATP-dependent manner.

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Introduction: Dysfunction of mitochondrial complex I causes hypersensitivity to volatile anesthetics (VAs) in nematodes, flies, mice and humans.[1-4] NDUFS4 (complex I subunit)-KO mouse shows hypersensitivity to VAs and is a well-established model for mitochondrial disease.[5] VAs inhibited presynaptic function of excitatory neurons; however, it remained unclear what is the precise mechanism by which VAs exert effects on synaptic neurotransmission.[6] Recent work showed that isoflurane inhibits mitochondrial complex I, reduces ATP levels at presynaptic terminals, and inhibits synaptic vesicle endocytosis.[7] During neuronal activity, calcium also plays an important role in both exocytosis and endocytosis of synaptic vesicles.[8] Here, we investigated the effects of isoflurane on mitochondrial regulation of calcium levels in presynaptic terminals and whether rescue of ATP defects would alleviate any changes in calcium levels.

Methods: We performed live-cell optical imaging to measure changes in calcium levels during neuronal stimulations in presynaptic terminals from hippocampal neuronal cultures. Primary hippocampal cells from mouse pups (P0 or P1) were cultured and transfected with a construct containing VGlut1-GCaMP5 (to measure synaptic calcium levels). A CRE-recombinase construct was used to generate *Ndufs4* (KO) cultures. Each cell was co-transfected with a mCherry-synaptophysin (to identify synaptic boutons). To study the changes of calcium levels in presynaptic boutons during neuronal activity, live-cell images were obtained in presynaptic terminals at DIV 10–14 after transfection. Intense stimulations were elicited at 10Hz for 60s and images were acquired using an acquisition rate of once ever 10 seconds (0.1Hz). To analyze the fluorescence response to stimulation over time, the background-subtracted fluorescence at each time point was normalized to the total amount of fluorescence evoked by the stimulation. Decay times were calculated by fitting a first order exponential curve.

Results: Both WT and *Ndufs4*(KO) cultures showed increased calcium levels followed by a return to baseline (or overshoot) during stimulations in the absence of isoflurane (Not shown). However, exposure to isoflurane treatment at whole animal $2XEC_{50}$s slowed the return to baseline (measured by decay times) of calcium levels following the 2nd stimulation in boutons from both wildtype (**Figure, Table 1**) and *Ndufs4* (KO) (**Figure, Table 2**) cultures compared to unexposed controls. The addition of 30mM glucose alleviated the delayed return to baseline of calcium levels in the presence of isoflurane from both genotypes (**Figure, Tables 1,2**).

Conclusions: Isoflurane treatment inhibited a calcium removal during neuronal activity in the presynaptic boutons from both WT and *Ndufs4* (KO) cultures. Notably, lower concentrations of isoflurane (0.6%) inhibit the *Ndufs4(KO)* (**Figure, Table 2**) but not wildtype neurons (**not shown**). Previous work showed that the 0.6% isoflurane causes decreased ATP levels in the KO but not in wildtype and that ATP levels are rescued by 30mM glucose levels.[7] Here we find that 30mM glucose also rescued the inhibition of calcium removal, similar to the alleviation of the inhibition of synaptic endocytosis caused by isoflurane. Since it is known that 30mM glucose rescues ATP levels in the presence of isoflurane, we interpret these changes to represent isoflurane-dependent inhibition of ATP-dependent calcium removal from the synapse. Calcium levels may be upstream of the defect in endocytosis caused by isoflurane. Whether inhibition of endocytosis is a result of failure of calcium removal or an independent effect remains to be determined. We are planning to investigate which calcium removal pathways are affected by isoflurane and whether the failure of calcium removal leads to the defect in endocytosis.

References: References. **1**. *Anesth*. 90**:**545, 1999. **2**. *PLoS One* 7:e42904, 2012. **3**. *Anesth Analg*. 133: 924, 2021. **4**. *Sci Rep.* 8: 2348, 2018. 5. *Cell Metab*. 7:312, 2008. 6. *Br J Anaesth.* 120:1019, 2018. 7. *Curr Biol.* 32:3016, 2022. **8.** *Proc Natl Acad Sci*. 119(20):e2111051119, 2022.

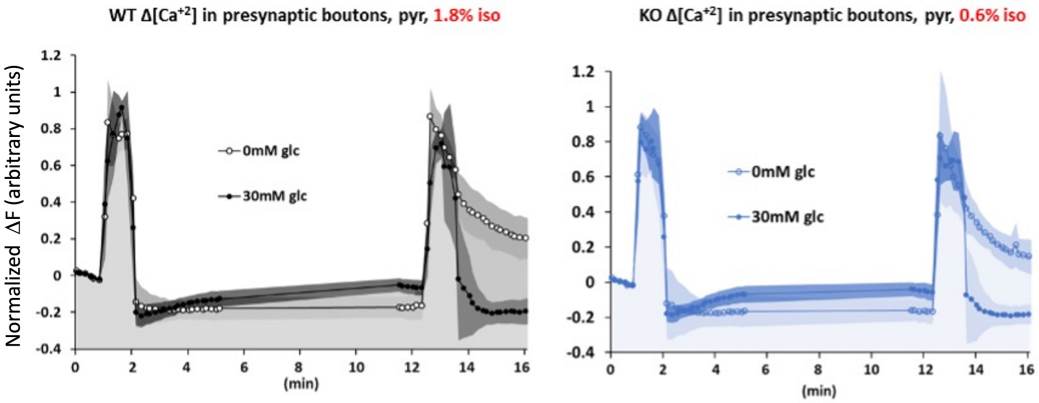

Figure. Changes in synaptic calcium levels following electrical stimulations of hippocampal cultures in the presence of isoflurane (1.8% for WT; 0.6% for KO) supplemented with pyruvate. 30mM glucose provoked a calcium removal during isoflurane exposure compared to no exogenous glucose. Left panel shows results in wildtype neurons at 1.8% isoflurane. Right panel shows results in *Ndufs4(KO)* neurons.

treatment	stim.	Decay time mean	SD	N	p values	t-test
(1) 0mM glucose, 0% isoflurane	1st	6.04	0.33	7		
	2nd	16.36	14.07	7		
(2) 0mM glucose, 1.8% isoflurane	1st	10.29	4.03	6	**0.017**	(1) vs (2)
	2nd	155.89	60.56	6	**0.0001**	
(3) 30mM glucose, 0% isoflurane	1st	6.06	0.64	6	0.84	(3) vs (4)
	2nd	8.20	6.25	6	0.25	
(4) 30mM glucose, 1.8% isoflurane	1st	6.31	2.91	8	0.052	**(2) vs (4)**
	2nd	15.59	13.92	8	**0.00003**	

Table 1. Isoflurane/glucose effects on wildtype decay times. Effects of isoflurane and glucose on decay times following neuronal stimulation (10Hz, 60 seconds). Note the increased decay time following the second stimulations in the presence of isoflurane (2) which was alleviated by the addition of 30mM glucose.

treatment	stim.	Decay time mean	SD	N	p values	t-test
(1) 0mM glucose, 0% isoflurane	1st	9.47	6.09	6		
	2nd	28.03	20.59	6		
(2) 0mM glucose, 0.6% isoflurane	1st	12.37	11.08	8	0.58	(1) vs (2)
	2nd	146.92	98.89	8	**0.014**	
(3) 30mM glucose, 0% isoflurane	1st	5.99	0.80	8	0.51	(3) vs (4)
	2nd	10.47	9.34	8	0.36	
(4) 30mM glucose, 0.6% isoflurane	1st	6.35	1.23	8	0.15	**(2) vs (4)**
	2nd	16.7	16.23	8	**0.0025**	

Table 2. Isoflurane/glucose effects on *Ndufs4(KO)* decay times. Effects of isoflurane and glucose on decay times following neuronal stimulation (10Hz, 60 seconds). Note the increased decay time following the second stimulations in the presence of 0.6% isoflurane (2) which was alleviated by the addition of 30mM glucose.