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Glutamatergic neurotransmission links sensitivity to volatile anesthetics with mitochondrial function

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Summary

An enigma of modern medicine has persisted for over 150 years. The mechanisms by which volatile anesthetics (VAs) produce their effects (loss of consciousness, analgesia, amnesia, immobility) remain an unsolved mystery. Many attractive putative molecular targets have failed to produce a significant effect when genetically tested in whole animal models [1–3]. However, mitochondrial defects increase VA sensitivity in diverse organisms from nematodes to humans [4–6]. *Ndufs4* knock-out (KO) mice lack a subunit of mitochondrial complex I and are strikingly hypersensitive to VAs, yet resistant to the intravenous anesthetic ketamine [7]. The change in VA sensitivity is the largest reported for a mammal. Limiting *NDUFS4* loss to a subset of glutamatergic neurons recapitulates the VA hypersensitivity of *Ndufs4(KO)* mice, while loss in GABAergic or cholinergic neurons does not. Baseline electrophysiologic function of CA1 pyramidal neurons does not differ between *Ndufs4(KO)* and controls. Isoflurane concentrations that anesthetize only *Ndufs4(KO)* mice (0.6%) decreased the frequency of spontaneous excitatory postsynaptic currents (sEPSCs) only in *Ndufs4(KO)* CA1 neurons, while concentrations effective in control mice (1.2%) decreased sEPSC frequencies in both control and *Ndufs4(KO)* CA1 pyramidal cells. Spontaneous inhibitory postsynaptic frequencies (sIPSCs) were not differentially affected between genotypes. The effects of isoflurane were similar on evoked field excitatory

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Author Contributions

PIZ and CBW designed and performed electrophysiologic experiments, evaluated results, contributed to the writing of the manuscript. AQ designed and performed genetic experiments, evaluated results, contributed to the writing of the manuscript. JMR designed electrophysiologic experiments, evaluated results and contributed to the writing of the manuscript. PGM designed experiments, performed genetic experiments, evaluated results, contributed to the writing of the manuscript. MMS designed experiments, evaluated results, and contributed to the writing of the manuscript.

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postsynaptic potentials (fEPSPs) and paired pulse facilitation (PPF) in KO and control hippocampal slices. We propose that CA1 pre-synaptic excitatory neurotransmission is hypersensitive to isoflurane in *Ndufs4(KO)* due to inhibition of pre-existing reduced complex I function, reaching a critical reduction that can no longer meet metabolic demands.

eTOC blurb

Ndufs4(KO) mice are very hypersensitive to volatile anesthetics. Zimin et al show that VGLUT2 specific *Ndufs4(KO)* recapitulated the phenotype while GABAergic and cholinergic *Ndufs4(KO)* did not. Isoflurane selectively inhibited excitatory neurotransmission in the CA1 of the hippocampus of *Ndufs4(KO)* at a dose that did not affect controls.

Results and Discussion

Complex I dysfunction in VGLUT2-expressing neurons recapitulates hypersensitivity to isoflurane and halothane observed in the total KO mice

To understand the cell specificity of mitochondrial complex I dysfunction and anesthetic hypersensitivity, we investigated the sensitivity to isoflurane and halothane of control mice with that of mice with *Ndufs4* knocked out selectively in GABAergic neurons (GABA-specific KO mice), VGLUT2-positive glutamatergic neurons (VGLUT2-specific KO mice) or cholinergic neurons (CHAT-specific KO mice). VGLUT2-specific KO mice were markedly hypersensitive to isoflurane and halothane, similar to the total KO mice. GABA-specific and CHAT-specific KO mice were not hypersensitive to either halothane or isoflurane, except for a small increase in sensitivity of CHAT-specific KO mice to halothane (Figure 1). These results support the hypothesis that excitatory glutamatergic transmission is the major contributor to volatile anesthetic hypersensitivity of KO mice.

Complex I dysfunction does not affect intrinsic membrane properties and synaptic baseline activity in hippocampal CA1 pyramidal neurons

To understand the link between mitochondrial complex I dysfunction and anesthetic hypersensitivity, we investigated intrinsic and synaptic properties of hippocampal CA1 pyramidal neurons in KO animals at baseline and following exposure to isoflurane. The CA1 region is a well-characterized region whose response to a multitude of anesthetics has been extensively studied, and which also has been implicated in the function of VAs [8–12]. We found no significant differences in the intrinsic membrane properties of hippocampal CA1 pyramidal neurons in slices obtained from control and KO mice. The resting membrane potential was -61.03 ± 1.24 mV and -62.33 ± 1.05 mV for control and KO cells, respectively. Cellular input resistance was 152.39 ± 6.25 M Ω and 148.28 ± 5.58 M Ω for control and KO cells, respectively.

No differences were found in sEPSC frequency, amplitude or decay time between genotypes (Figure 2A). There were no significant differences between KO and control neurons in miniature excitatory post-synaptic current mEPSC frequency, amplitude, or decay time at baseline (Figure 2B).

There were no differences in sIPSC frequency, amplitude and decay time between KO and control neurons (Figure 2C). Finally, there were no differences in miniature inhibitory post-synaptic current (mIPSC) frequency, amplitude and decay time between KO and control neurons (Figure 2D).

These results indicate that neuronal function in CA1 hippocampal pyramidal cells was not different between control and KO mice in the absence of anesthetic. Thus, hypersensitivity to isoflurane in KO mice is not due to a generalized metabolic deficiency in basic neuronal function. The specific resistance of the mutant to ketamine also supports the conclusion that a general metabolic deficiency is unlikely the explanation for the hypersensitivity to volatile anesthetics [7].

Our recordings were made before any symptoms or histopathologic changes develop in the mutant, and from a region that is relatively spared from degeneration. The fact that most parameters studied failed to show differences between control and KO cells at baseline is consistent with the fact that ATP concentrations in muscle and blood are not decreased in the mutant [13]. In fact, the respiratory capacity of intact mitochondria in the KO is 50 – 100 % of the wild-type depending on the complex I substrate [14]. Additional sources of ATP such as glycolysis could also compensate to some degree in order to maintain network activity at baseline.

CA1 sEPSC frequency is hypersensitive to 0.6% isoflurane in KO neurons

We next studied the effects of mitochondrial dysfunction on synaptic activity with isoflurane exposure. Exposure to 0.6% isoflurane significantly decreased the sEPSC frequency of KO cells without changing the sEPSC frequency of control neurons (Figure 3A,B). Moreover, sEPSC frequency in KO neurons did not fully revert to pre-exposure levels after 15 min of wash with artificial cerebrospinal fluid (ACSF). Interestingly, sEPSC amplitudes and decay times were not affected by 0.6% isoflurane exposure in either control or KO neurons (Figure 3C,D). In the presence of 1.2% isoflurane sEPSC frequency was significantly reduced in both KO and control neurons (Figure 3A,B). As was the case for 0.6% isoflurane, sEPSC frequency did not fully recover after 15 min of wash in KO cells while sEPSC frequency of control neurons returned to the level prior to exposure (Figure 3B). In order to rule-out a possible isoflurane-independent run-down, control experiments were performed in which KO cells were voltage-clamped and sEPSC frequency was monitored for the duration of the isoflurane treatment experiment. No significant change in sEPSC frequency was observed (data not shown). Exposure to 1.2% isoflurane did not affect sEPSC amplitude or decay time in either control or KO cells (Figure 3C,D). Application of 0.6% isoflurane did not cause any changes in mEPSC frequency, amplitude or decay time in either genotype (Figure 3E–H).

Isoflurane caused a specific inhibition of sEPSC frequency only in mutant cells at a dose that anesthetized mutant animals but not controls. Control cells showed an identical response at a dose that approximated the EC_{50} of isoflurane *in vivo* for control animals. This implies that the same phenomenon underlies the effect of isoflurane on synaptic transmission in both genotypes, and determines the *in vivo* response.

Interestingly, sEPSC frequency in KO cells did not return to baseline following exposure to isoflurane. It is unlikely that isoflurane irreversibly binds to its target since anesthetized KO mice awoken within 5–10 minutes after removal from 0.4% isoflurane. Additionally, membrane potential was maintained throughout cellular recordings supporting the conclusion that cell death is not the cause of irreversibility of isoflurane-induced sEPSC frequency decrease. Since isoflurane is non-detectable in the superfusion-recording chamber after 15 minutes of wash as measured by gas chromatography, our results could be explained by inability of mitochondria to recover ATP levels within the time frame of experiment. Additional studies are required to provide mechanistic insights for this phenomenon.

Studies of the effects of isoflurane on sEPSC parameters on slices from GABAergic and cholinergic specific KO mice did not show differences between mutant and control slices. Although frequencies decreased in both mutant and control lines, this change did not reach significance (Figure S1). Additional effectors may exist for synaptic responses to isoflurane.

Isoflurane increases CA1 sIPSC decay time similarly in KO and in control neurons

sIPSC frequency was not affected by 0.6% isoflurane in either KO or control genotypes (Figure S2A,B). Exposure to 0.6% isoflurane slightly decreased sIPSC amplitude in control cells without an effect on sIPSC amplitude in KO cells (Figure S2C). Application of 0.6% isoflurane increased sIPSC decay time similarly in control and KO neurons, which returned to pre-exposure levels after 15 minutes of wash with ACSF (Figure S2D). Application of 1.2% isoflurane did not affect sIPSC frequency and amplitude of either genotype tested (Figure S2A–C). Exposure to 1.2% isoflurane increased sIPSC decay time equally in control and KO neurons (Figure S2D), which returned to pre-exposure levels with 15 minutes of wash.

mIPSC frequency was not affected by 0.6% isoflurane exposure in either control or KO cells (Figure S2E,F). mIPSC amplitude decreased slightly, but statistically significantly, following exposure to 0.6% isoflurane (Figure S2G). mIPSC amplitude after wash was not significantly different from pre-exposure levels. mIPSC decay time increased after 0.6% isoflurane exposure in both genotypes similarly and returned to pre-exposure level after wash (Figure S2H).

An anesthetic induced increase in sIPSC decay time is consistent with previous reports [15], interpreted to be a result of isoflurane interacting with GABA_A receptors directly. Mitochondrial complex I dysfunction did not affect sIPSC parameters at baseline. sIPSC amplitude demonstrated a small decrease after 0.6% isoflurane exposure in control, but not in KO cells. Since mIPSC data did not show any differential effect after 0.6% isoflurane exposure, the differential effect of 0.6% isoflurane on sIPSC amplitude could be due to differences in GABAergic neuronal spiking. However, the magnitude of the effect makes it unlikely to be of biological significance. These results indicate that hippocampal inhibitory circuitry is not affected by mitochondrial complex I dysfunction, and that the differential signaling in CA1 neurons between mutant and control cells is not the result of increased upstream inhibitory signaling. The lack of effect of the loss of NDUFS4 in GABAergic neurons on sensitivity to isoflurane and halothane as measured by tail clamp also supports this conclusion.

Isoflurane-induced depression of field excitatory postsynaptic potentials is larger in KO slices than in control slices

We next studied the effects of isoflurane on evoked field excitatory postsynaptic potentials (fEPSPs) and paired pulse facilitation (PPF) in KO and control hippocampal slices (Figure 4A). At baseline PPF ratios were similar for KO and control slices (Figure 4B–D). The PPF ratios were not significantly different at various inter-pulse intervals ranging from 10–100 ms between control and KO slices (Figure S3).

Application of 0.6% isoflurane increased the PPF ratio of both control and KO slices similarly (Figure 4B,F). In addition, 0.6% isoflurane reversibly decreased the amplitudes of first and second fEPSP similarly for control and KO slices (Figure 4B,D). Application of 1.2% isoflurane increased the PPF ratio of both control and KO slices similarly (Figure 4C,F). In addition, 1.2% isoflurane depressed both first and second fEPSPs to a greater extent than 0.6% isoflurane and depressed fEPSPs in KO slices more than in control slices (Figure 4C–E).

An increase in PPF ratio concurrent with a depression of fEPSP amplitude after exposure to VAs has been reported [16, 17], and has been interpreted as evidence for presynaptic depression of calcium-dependent glutamate release. In this report we observed a larger depression of fEPSP amplitude in KO neurons following the exposure to 1.2% isoflurane. This result, coupled with data in Figure 1, further suggests that glutamatergic synapses are affected by the mitochondrial complex I dysfunction, probably due to their high energetic demand upon stimulation. This finding is consistent with the study by Pathak *et al.* that reported that electrical stimulation-induced depression of ATP concentration in synaptic boutons is larger in KO cells than in WT cells [18]. The fiber volleys – a measure of a number of action potentials arriving to nerve terminals – were not affected by isoflurane at either concentration tested (data not shown). This result indicates that the fEPSP depression could not be explained by attenuation of action potential conduction in KO slices.

Depression of first and second fEPSP amplitudes after isoflurane exposure appears to contradict whole-cell patch-clamp data, where we did not see any changes to sEPSC amplitudes. This finding could be explained by increased energetic demand of nearly synchronous recruitment of a large number of fibers in extracellular recordings.

Conclusions

We have discovered that the VA hypersensitivity of KO mice is recapitulated when the gene is lost in the distribution of the *Vglut2* promoter. Electrophysiologic studies reveal that the characteristics of the CA1 of the hippocampus are not changed in the mutant, except when exposed to isoflurane. A low concentration of isoflurane decreased the frequency of sEPSCs only in the mutant.

Since mEPSCs of KO neurons were not affected by 0.6% isoflurane, it is possible that isoflurane reduced the network input into CA1 pyramidal cells, rather than synaptic transmission within the CA1 region. Our results with sIPSCs and with field potentials make this explanation unlikely. Isoflurane has also been demonstrated to inhibit vesicle exocytosis

through reduced Ca^{2+} influx specifically in excitatory synapses [19]. Ca^{2+} influx could be differentially affected in KO neurons in response to isoflurane, thereby explaining specific inhibition of sEPSC frequency following 0.6% isoflurane exposure. Alternatively, KO excitatory synapses, when exposed to isoflurane, may lack the ability to meet the increased energetic demands of spontaneous firing compared to the resting state of mEPSCs. It will therefore be useful to explore the electrophysiological effects of *Ndufs4* mutation under states of high energetic demand.

There are several energy-demanding processes of neuronal signaling, including reversal of Na^+ entry for action potentials, reversal of Na^+ entry at resting potential, reversal of ion fluxes through postsynaptic receptors, glutamate recycling, and reversal of presynaptic Ca^{2+} entry [20]. Since the fiber volley amplitude was not affected after 0.6% or 1.2% isoflurane exposure in KO slices, it is unlikely that inhibition of reversal of Na^+ entry for action potentials is the mechanism explaining hypersensitivity of KO animals to volatile anesthetics. Inhibition of any remaining energy-demanding processes could explain the hypersensitivity of KO animals.

Mitochondria are increasingly appreciated by a broad audience as key regulators of neuronal activity, yet their role in the state of anesthesia is not clear. It has long been known that VAs specifically inhibit complex I function in isolated mitochondria from worms and mammals [21, 22]. Our work in worms, mice and children corroborated these *in vitro* findings implicating complex I, compared to other mitochondrial components, as uniquely controlling anesthetic sensitivity [6, 7, 23]. Taken together, these data link a very novel target of VAs to a specific facet of normal neuronal function and suggest that glutamatergic neurotransmission is selectively sensitive to isoflurane in the mutant. We hypothesize that VA inhibition of complex I in a mutant with compromised complex I function may deplete ATP levels such that presynaptic function is limited in the CA1 and perhaps elsewhere, as glutamatergic cells no longer match energetic demand with supply. This same pathway could exist in animals with normal complex I function, but require higher concentrations of isoflurane to achieve limiting amounts of ATP.

Several questions remain. We cannot yet explain how our current data relate to the resistance of the animal to ketamine, although it is now clear that this drug has many possible targets [24]. Also, we have not ruled out that mitochondria may be a *de novo* target for VAs in the mutant, although the main electrophysiologic change we measure in isoflurane occurs at a dose that approximates the EC_{50} of the drug for the whole animal phenotype. We infer that the simplest model is that mitochondria are in fact a target of VAs, and that inhibition of specific circuit elements, energy dependent excitatory transmission, is a mechanism that underlies sensitivity to VAs in the KO.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- VGLUT2-specific loss of NDUFS4 causes whole animal anesthetic hypersensitivity
- Isoflurane depressed hippocampal sEPSC frequencies selectively in *Ndufs4(KO)*
- sIPSCs were not affected by NDUFS4 loss
- The *Ndufs4* mutation selectively affects presynaptic function in excitatory neurons

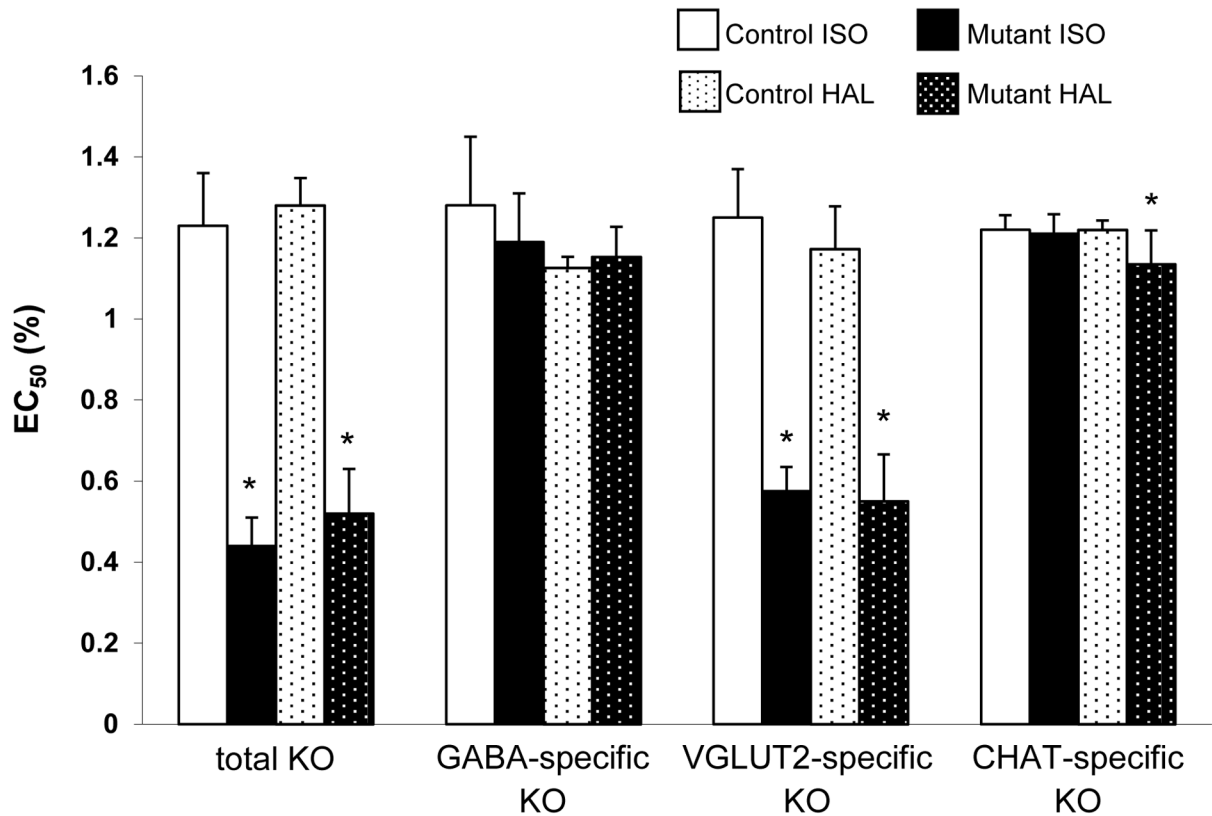
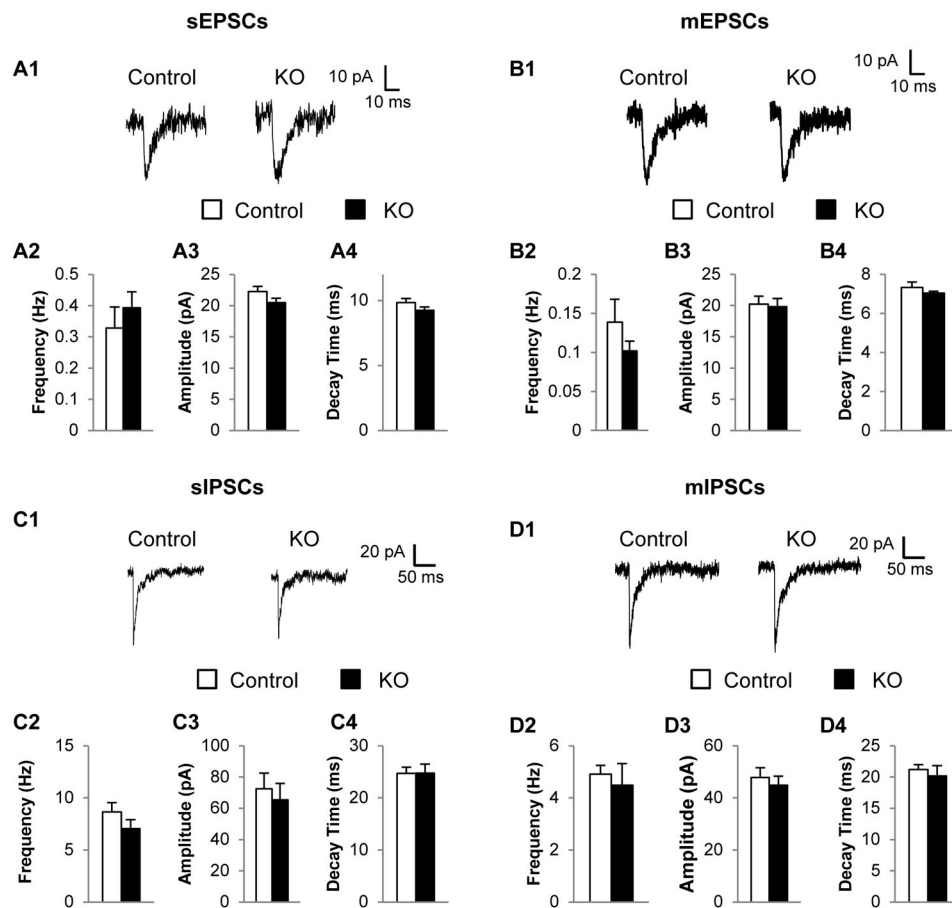


Figure 1.

VGLUT2-specific KO mice show hypersensitivity for halothane and isoflurane similar to global KO mice. While GABA-specific KO and CHAT-specific KO mice show similar EC₅₀s for isoflurane (ISO) and halothane (HAL) to control mice, VGLUT2-specific KO mice demonstrate hypersensitivity to both anesthetics. Data for the total KO mice was published before [7] and shown here for comparison. Anesthetic concentrations are expressed in % by volume. Graph bars represent mean, error bars represent standard deviation. EC₅₀s for isoflurane and halothane were reduced in VGLUT2-specific KO mice ($P < 0.001$ for both isoflurane and halothane, global control: $n = 10$ mice for isoflurane, $n = 6$ mice for halothane, global KO: $n = 10$ mice for isoflurane, $n = 6$ mice for halothane, VGLUT2-specific control: $n = 5$ mice for isoflurane, $n = 7$ mice for halothane, VGLUT2-specific KO: $n = 5$ mice for isoflurane and for halothane). EC₅₀s for isoflurane and halothane were not significantly different between GABA-specific KO mice and corresponding control mice ($P = 0.176$ for isoflurane, $P = 0.585$ for halothane. GABA-specific control: $n = 6$ mice for isoflurane, $n = 5$ mice for halothane, GABA-specific KO: $n = 6$ mice for isoflurane and halothane). While EC₅₀s for isoflurane were not significantly different between CHAT-specific KO mice and their controls ($P = 0.420$. CHAT-specific control: $n = 6$ mice, CHAT-specific KO: $n = 5$ mice), there was a small statistically significant difference in EC₅₀s for halothane ($P = 0.034$. CHAT-specific control: $n = 10$ mice, CHAT-specific KO: $n = 7$ mice). See also Table S1.

**Figure 2.**

Effect of mitochondrial complex I dysfunction on excitatory and inhibitory synaptic function in hippocampal CA1 pyramidal neurons at baseline. **(A1)** Representative individual sEPSC event traces of control and KO neurons. **(A2–A4)** Quantification of absolute sEPSC frequency, amplitude, and decay time in control and KO cells (Control: $n = 50$ cells, KO: $n = 81$ cells). No statistically significant changes were observed in frequency ($P = 0.118$), amplitude ($P = 0.043$) or decay time ($P = 0.066$) between control and KO cells. **(B1)** Representative individual mEPSC event traces of control and KO neurons. **(B2–B4)** Quantification of absolute mEPSC frequency, amplitude, and decay time in control and KO cells (Control: $n = 10$ cells, KO: $n = 14$ cells). No significant differences were observed in mEPSC frequency ($P = 0.364$), amplitude ($P = 0.930$) or decay time ($P = 0.334$) between control or KO cells. **(C1)** Representative individual sIPSC event traces of control and KO neurons. **(C2–C4)** Quantification of absolute sIPSC frequency, amplitude, and decay time in control and KO cells (Control: $n = 21$ cells, KO: $n = 18$ cells). No significant differences were observed in sIPSC frequency ($P = 0.190$), amplitude ($P = 0.231$) or decay time ($P = 0.811$) between control or KO cells. **(D1)** Representative individual mIPSC event traces of control and KO neurons. **(D2–D4)** Quantification of absolute mIPSC frequency, amplitude, and decay time in control and KO cells ($n = 7$ cells). No significant differences were observed in mIPSC frequency ($P = 0.209$), amplitude ($P = 0.383$) or decay time ($P = 1.000$)

between control and KO cells. Graph bars here and in subsequent figures represent mean, error bars represent standard error of the mean.

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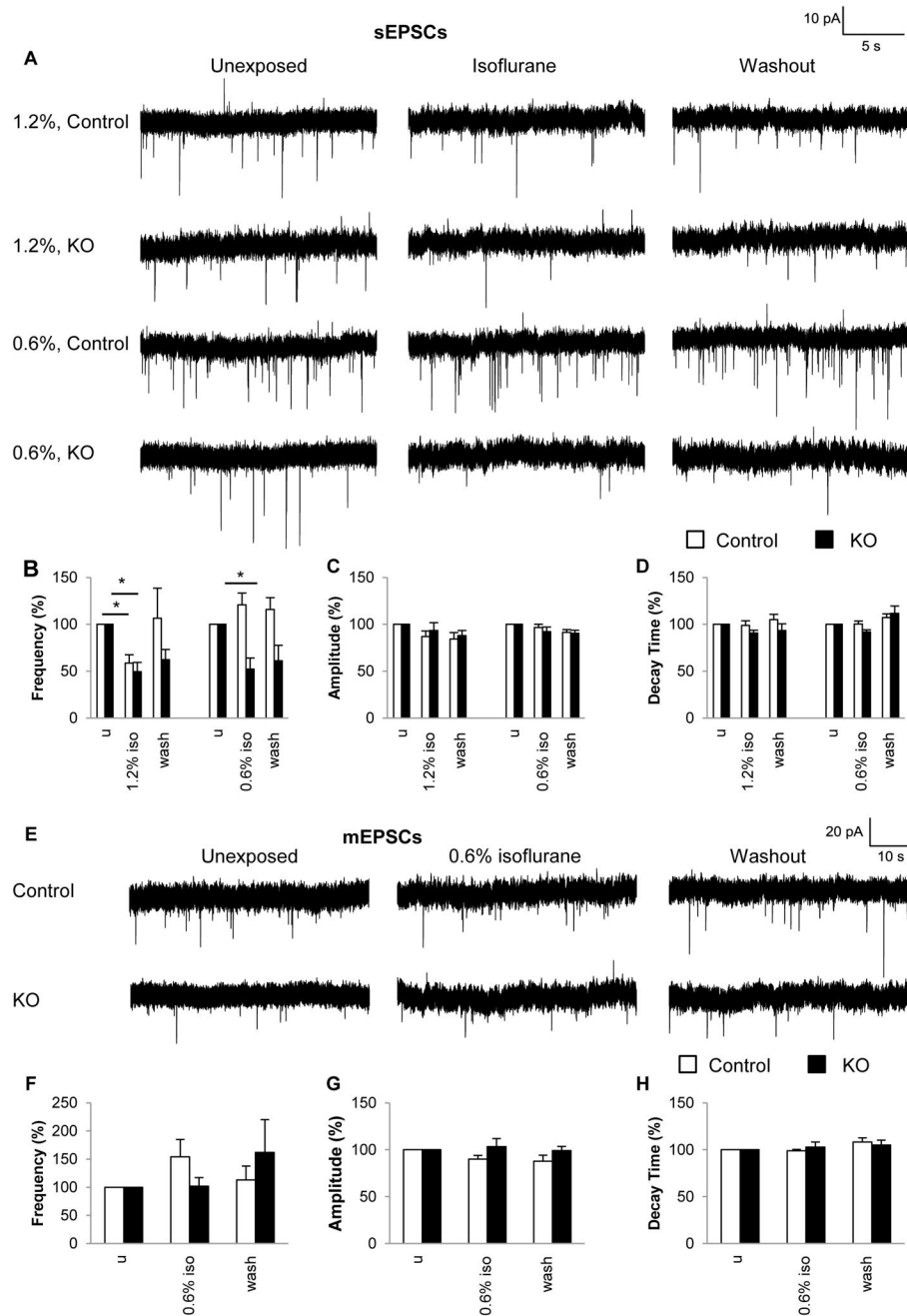


Figure 3. sEPSC frequency is more sensitive to isoflurane in hippocampal CA1 pyramidal neurons of mitochondrial complex I mutant. (A) Representative sEPSC traces before isoflurane exposure (Unexposed), during isoflurane exposure (Isoflurane), and after washout (Washout). Isoflurane concentration and genotype are shown on the left. (B–D) Quantification of relative sEPSC frequency, relative amplitude, and relative decay time (1.2% isoflurane control $n = 10$ cells, KO $n = 14$ cells, 0.6% isoflurane control $n = 13$ cells, KO 14 cells). 1.2% isoflurane significantly decreased sEPSC frequency of both control cells

($P < 0.05$) and KO cells ($P < 0.05$). 1.2% isoflurane exposure did not affect sEPSC decay time (Control: $P = 0.569$, KO: $P = 0.057$) or amplitude (Control: $P = 0.569$, KO: $P = 0.685$) of both control and KO cells. 0.6% isoflurane exposure did not affect sEPSC frequency recorded from control cells ($P = 0.794$), but significantly decreased sEPSC frequency recorded from KO cells ($P < 0.01$). 0.6% isoflurane exposure did not affect sEPSC decay time (Control: $P = 0.199$, KO: $P = 0.071$) or amplitude (Control: $P = 0.146$, KO: $P = 0.062$) of both control and KO cells. **(E)** Representative mEPSC traces before isoflurane exposure (Unexposed), during 0.6% isoflurane exposure (0.6% Isoflurane), and after washout (Washout). **(F–H)** Quantification of relative mEPSC frequency, relative amplitude, and relative decay time (Control: $n = 10$ cells, KO: $n = 10$ cells). 0.6% isoflurane exposure did not affect mEPSC frequency (Control: $P = 0.301$, KO: $P = 0.598$), amplitude (Control: $P > 0.05$, KO: $P = 0.670$), or decay time (Control: $P > 0.05$, KO: $P = 1.000$) of both control and KO cells. Relative frequency, amplitude and decay time here and in subsequent figures were normalized to the average corresponding parameter prior to isoflurane exposure for each individual trace and expressed in %. Here and in subsequent figures u denotes unexposed, iso denotes isoflurane, wash denotes washout. * indicates statistically significant difference with $P < 0.05$, for 0.6% isoflurane sEPSC data * indicates $P < 0.01$ per Bonferroni correction. See also Figures S1 and S2.

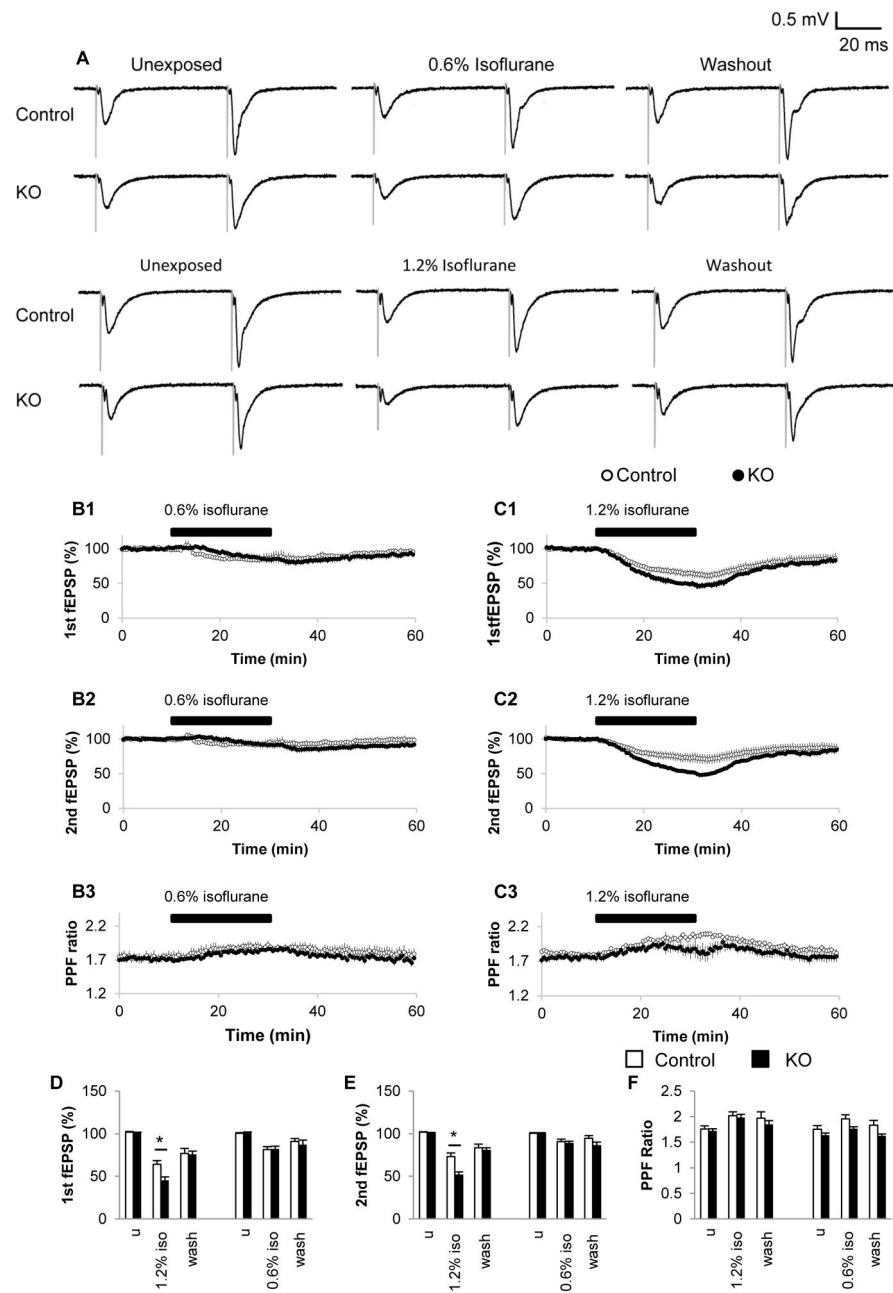


Figure 4.

Additional depression of 1st and 2nd fEPSP amplitude upon exposure to 1.2% isoflurane in mitochondrial complex I mutant recorded from Schaffer collateral – CA1 synapses. (A) Representative field recording traces before isoflurane exposure (Unexposed), during isoflurane exposure (Isoflurane), and after washout (Washout). Stimulation artefacts are truncated and shown in grey. (B1–B3) Time course of 0.6% isoflurane on 1st normalized fEPSP, 2nd normalized fEPSP, PPF ratio. (C1–C3) Time course of 1.2% isoflurane exposure on 1st normalized fEPSP amplitude, 2nd normalized fEPSP amplitude, PPF ratio. (D–F) Quantification of 1st normalized fEPSP amplitude, 2nd normalized fEPSP amplitude, PPF

ratio. Black horizontal bar represents isoflurane exposure. No difference was noted in PPF ratio between control and KO slices ($P = 0.163$, $n = 20$ and 21 slices). 0.6% isoflurane exposure decreased 1st fEPSP amplitude (Control: $P < 0.05$, KO: $P < 0.05$) and 2nd fEPSP amplitude (Control: $P < 0.05$, KO: $P < 0.05$), while increased PPF ratio (Control: $P < 0.05$, KO: $P < 0.05$) in both control and KO slices (Control: $n = 10$ slices, KO: $n = 10$ slices). 1.2% isoflurane exposure decreased 1st fEPSP amplitude (Control: $P < 0.05$, KO: $P < 0.05$) and 2nd fEPSP amplitude (Control: $P < 0.05$, KO: $P < 0.05$), while increased PPF ratio (Control: $P < 0.05$, KO: $P < 0.05$) in both control and KO slices (Control: $n = 11$ slices, KO: $n = 10$ slices). 1.2% isoflurane exposure produced larger depression of 1st fEPSP amplitude ($P = 0.005$) and 2nd fEPSP amplitude ($P = 0.002$) in KO slices compared to control slices. See also Figure S3.