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Title. Glutamatergic neurotransmission links sensitivity to volatile anesthetics with mitochondrial function

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Summary

A true 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 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.

Results and Discussion.

Rationale. To understand the link between mitochondrial complex I dysfunction and anesthetic hypersensitivity, we investigated anesthetic behavior of animals which have lost NDUFS4 in specific types of neurons. We also compared the synaptic properties of hippocampal CA1 pyramidal neurons in young 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]. Our findings demonstrate specific and significant differences between wildtype and KO mice in the effects of isoflurane on excitatory pathways.

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

We compared 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. 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 MΩ and 148.28 ± 5.58 MΩ 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 at baseline (Figure 2B).

There were no differences in sIPSC frequency, amplitude and decay between KO and control neurons (Figure 2C). Finally, there were no differences in miniature

inhibitory post-synaptic current (mIPSC) frequency, amplitude and decay 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 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 awaken 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.

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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References

1. Rudolph, U., and Antkowiak, B. (2004). Molecular and neuronal substrates for general anaesthetics. Nat. Rev. Neurosci. *5*, 709-720.
2. Hemmings, H.C., Jr. (2009). Sodium channels and the synaptic mechanisms of inhaled anaesthetics. Br. J. Anaesth. *103*, 61-69.
3. Sonner, J.M., and Cantor, R.S. (2013). Molecular mechanisms of drug action: an emerging view. Annu Rev Biophys *42*, 143-167.
4. Morgan, P.G., and Sedensky, M.M. (1994). Mutations conferring new patterns of sensitivity to volatile anesthetics in Caenorhabditis elegans. Anesthesiology *81*, 888-898.
5. Falk, M.J., Kayser, E.B., Morgan, P.G., and Sedensky, M.M. (2006). Mitochondrial complex I function modulates volatile anesthetic sensitivity in C. elegans. Curr Biol *16*, 1641-1645.
6. Morgan, P.G., Hoppel, C.L., and Sedensky, M.M. (2002). Mitochondrial defects and anesthetic sensitivity. Anesthesiology *96*, 1268-1270.
7. Quintana, A., Morgan, P.G., Kruse, S.E., Palmiter, R.D., and Sedensky, M.M. (2012). Altered anesthetic sensitivity of mice lacking Ndufs4, a subunit of mitochondrial complex I. PLoS One *7*, e42904.
8. Berg-Johnsen, J., and Langmoen, I.A. (1990). Mechanisms concerned in the direct effect of isoflurane on rat hippocampal and human neocortical neurons. Brain Res. *507*, 28-34.
9. MacIver, M.B., and Roth, S.H. (1988). Inhalation anaesthetics exhibit pathway-specific and differential actions on hippocampal synaptic responses in vitro. Br. J. Anaesth. *60*, 680-691.
10. Miu, P., and Puil, E. (1989). Isoflurane-induced impairment of synaptic transmission in hippocampal neurons. Exp. Brain Res. *75*, 354-360.
11. Pearce, R.A. (1996). Volatile anaesthetic enhancement of paired-pulse depression investigated in the rat hippocampus in vitro. J. Physiol. *492 (Pt 3)*, 823-840.
12. Richards, C.D., and White, A.E. (1975). The actions of volatile anaesthetics on synaptic transmission in the dentate gyrus. J. Physiol. *252*, 241-257.
13. Kruse, S.E., Watt, W.C., Marcinek, D.J., Kapur, R.P., Schenkman, K.A., and Palmiter, R.D. (2008). Mice with mitochondrial complex I deficiency develop a fatal encephalomyopathy. Cell Metab *7*, 312-320.
14. Johnson, S.C., Yanos, M.E., Kayser, E.B., Quintana, A., Sangesland, M., Castanza, A., Uhde, L., Hui, J., Wall, V.Z., Gagnidze, A., et al. (2013). mTOR inhibition alleviates mitochondrial disease in a mouse model of Leigh syndrome. Science *342*, 1524-1528.
15. Jones, M.V., and Harrison, N.L. (1993). Effects of volatile anesthetics on the kinetics of inhibitory postsynaptic currents in cultured rat hippocampal neurons. J. Neurophysiol. *70*, 1339-1349.
16. MacIver, M.B., Mikulec, A.A., Amagasu, S.M., and Monroe, F.A. (1996). Volatile anesthetics depress glutamate transmission via presynaptic actions. Anesthesiology *85*, 823-834.
17. Perouansky, M., Baranov, D., Salman, M., and Yaari, Y. (1995). Effects of halothane on glutamate receptor-mediated excitatory postsynaptic currents. A patch-clamp study in adult mouse hippocampal slices. Anesthesiology *83*, 109-119.
18. Pathak, D., Shields, L., Mendelsohn, B.A., Haddad, D., Lin, W., Gerencser, A.A., Kim, H., Brand, M.D., Edwards, R.H., and Nakamura, K. (2015). The Role of Mitochondrially Derived ATP in Synaptic Vesicle Recycling. J Biol Chem.
19. Baumgart, J.P., Zhou, Z.Y., Hara, M., Cook, D.C., Hoppa, M.B., Ryan, T.A., and Hemmings, H.C., Jr. (2015). Isoflurane inhibits synaptic vesicle exocytosis through

reduced Ca2+ influx, not Ca2+-exocytosis coupling. Proc. Natl. Acad. Sci. U. S. A. *112*, 11959-11964.

20. Harris, J.J., Jolivet, R., and Attwell, D. (2012). Synaptic energy use and supply. Neuron *75*, 762-777.
21. Kayser, E.B., Suthammarak, W., Morgan, P.G., and Sedensky, M.M. (2011). Isoflurane selectively inhibits distal mitochondrial complex I in Caenorhabditis elegans. Anesth Analg *112*, 1321-1329.
22. Harris, R.A., Munroe, J., Farmer, B., Kim, K.C., and Jenkins, P. (1971). Action of halothane upon mitochondrial respiration. Arch Biochem Biophys *142*, 435-444.
23. Kayser, E.B., Morgan, P.G., and Sedensky, M.M. (1999). GAS-1: a mitochondrial protein controls sensitivity to volatile anesthetics in the nematode Caenorhabditis elegans. Anesthesiology *90*, 545-554.
24. Sleigh J., Harvey.M., Voss L., Denny B. (2014). Ketamine - More mechanisms of action than just NMDA blockade. Trends in Anaesthesia and Critical Care *4*, 76-81.

Figure Legends

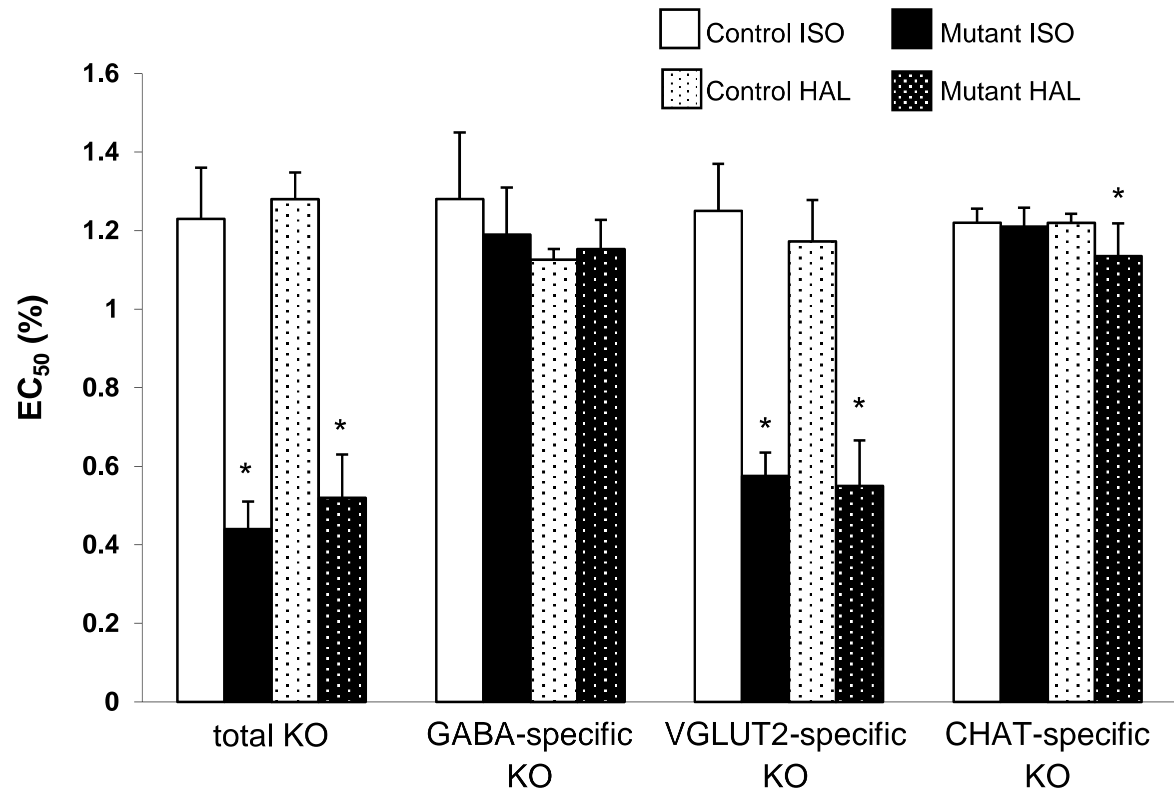
Figure 1. VGLUT2-specific KO mice show hypersensitivity for halothane and isoflurane similar to global KO mice. While GABA-specific KO and CHAT-specific KO mice show similar EC_{50}s for isoflurane (ISO) and halothane (HAL) to control mice, VGLUT2-specific KO mice demonstrate hypersensitivity to both 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. EC_{50}s 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). EC_{50}s 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 EC_{50}s 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 EC_{50}s 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) and decay time (P = 0.334) between control and 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) and decay time (P = 0.811) between control and 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) and 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.

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.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CHAT-specific KO

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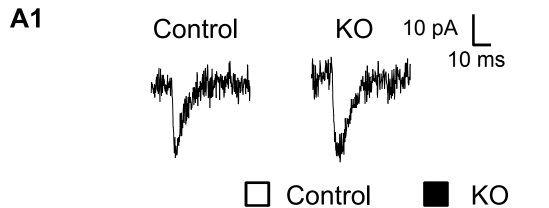
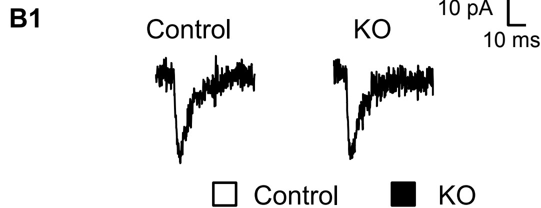
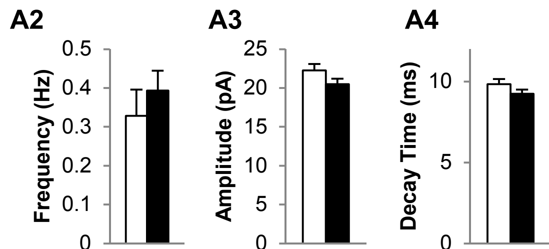
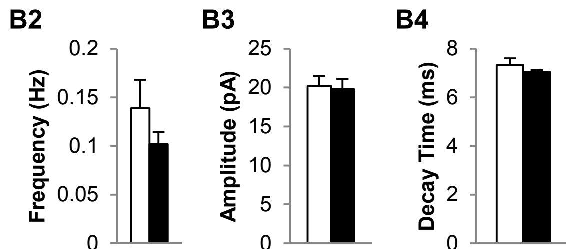
mEPSCs



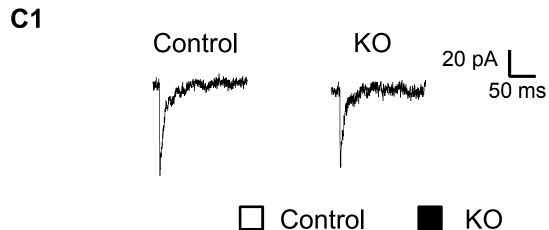


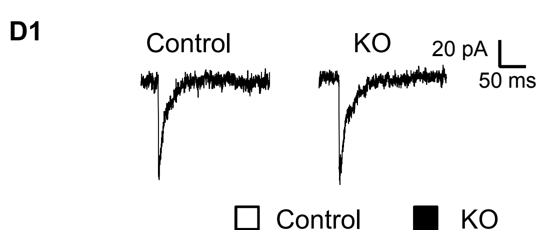


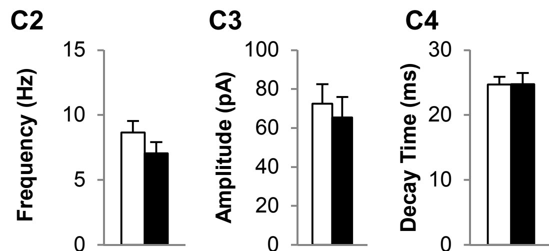


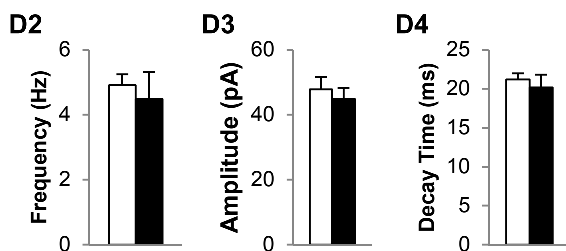
sIPSCs

mIPSCs









10 pA
5 s

sEPSCs

A

Unexposed | Isoflurane | Washout

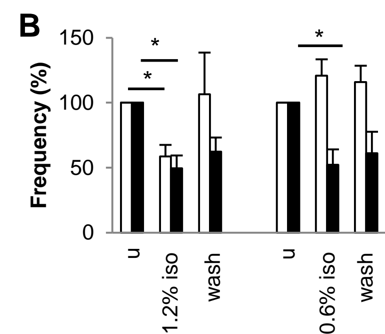
1.2%, Control

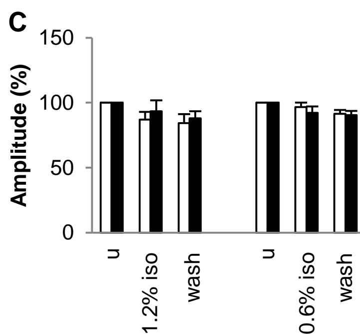
1.2%, KO

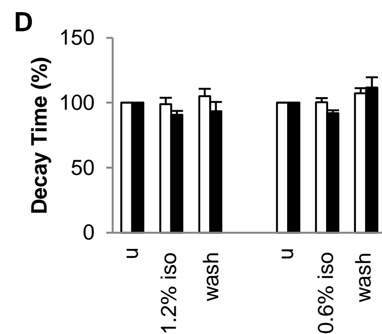
0.6%, Control

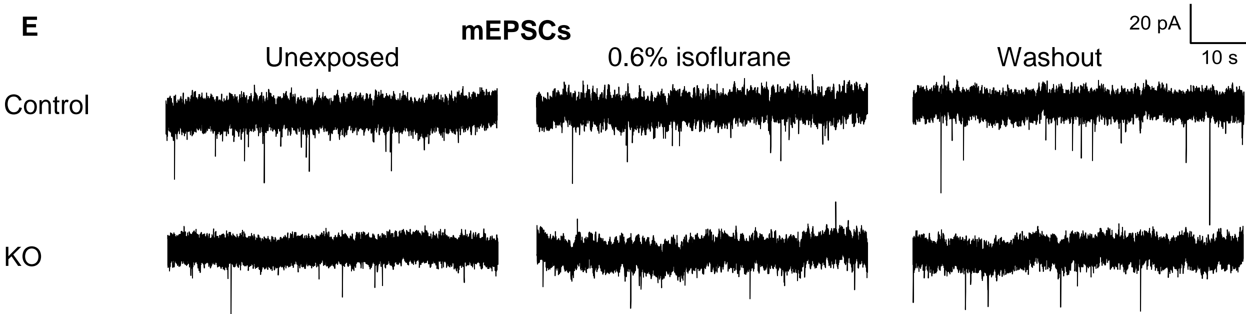
0.6%, KO

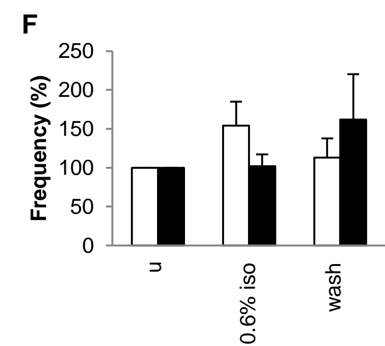
Control KO

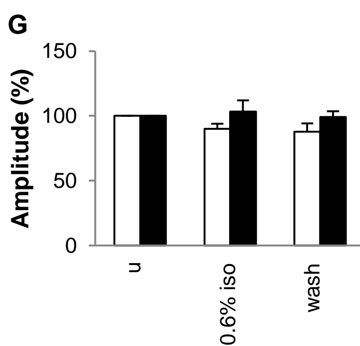


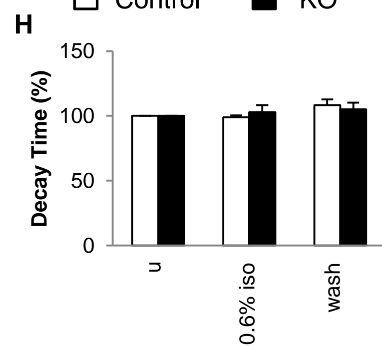


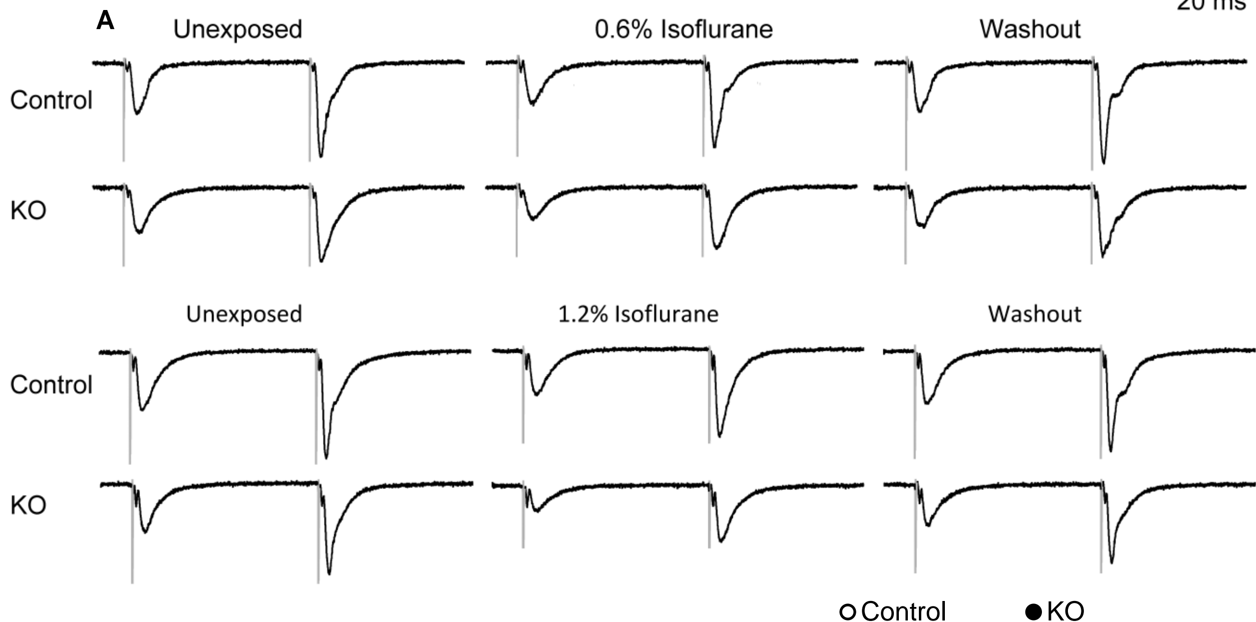




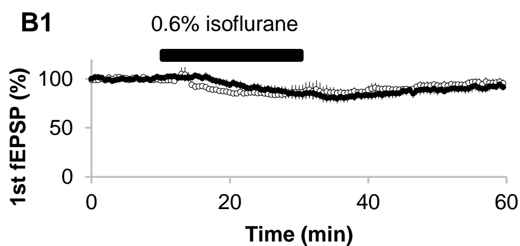


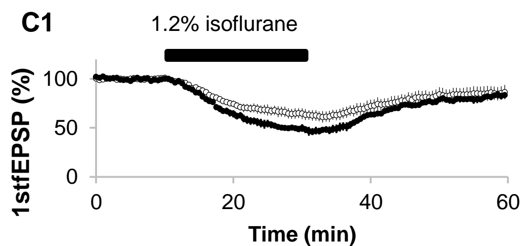


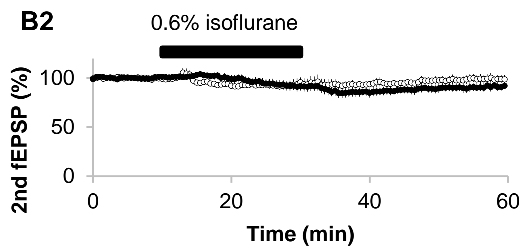


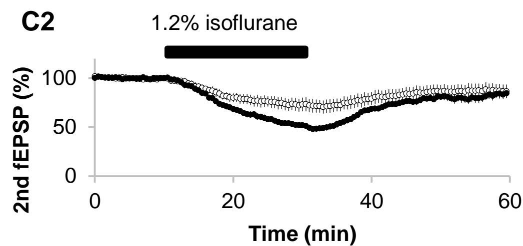


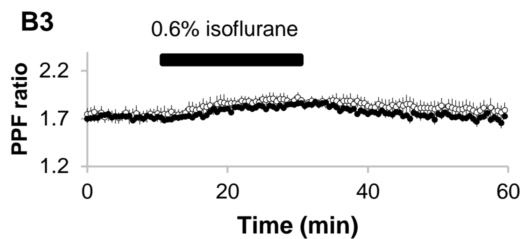
Control
KO

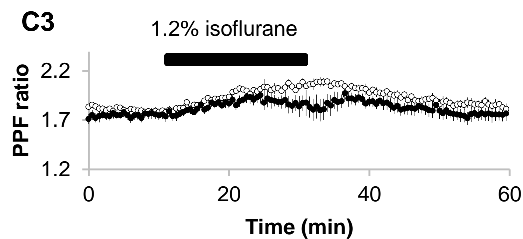




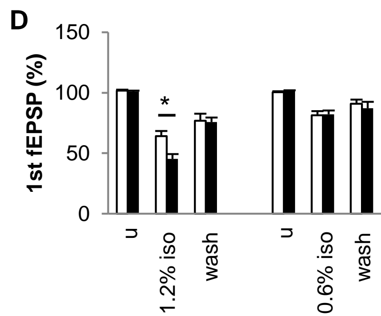


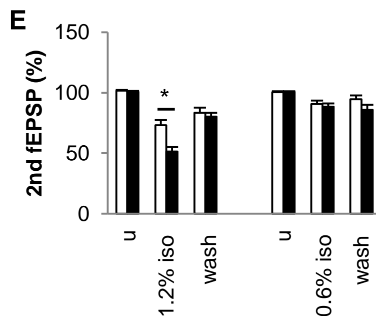


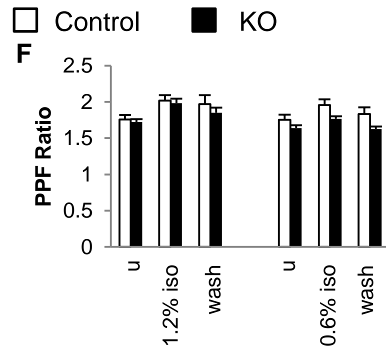




Control
KO







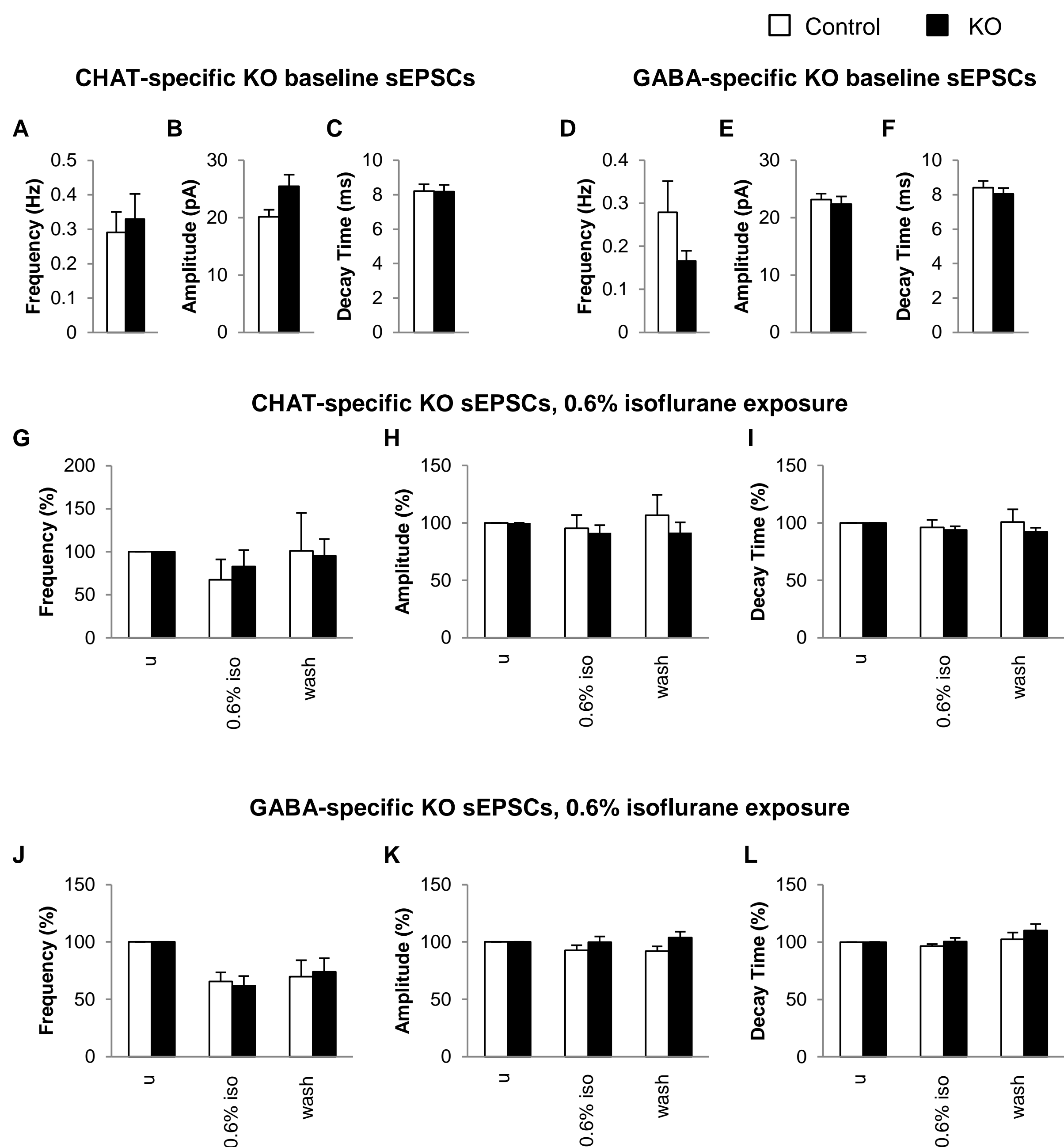
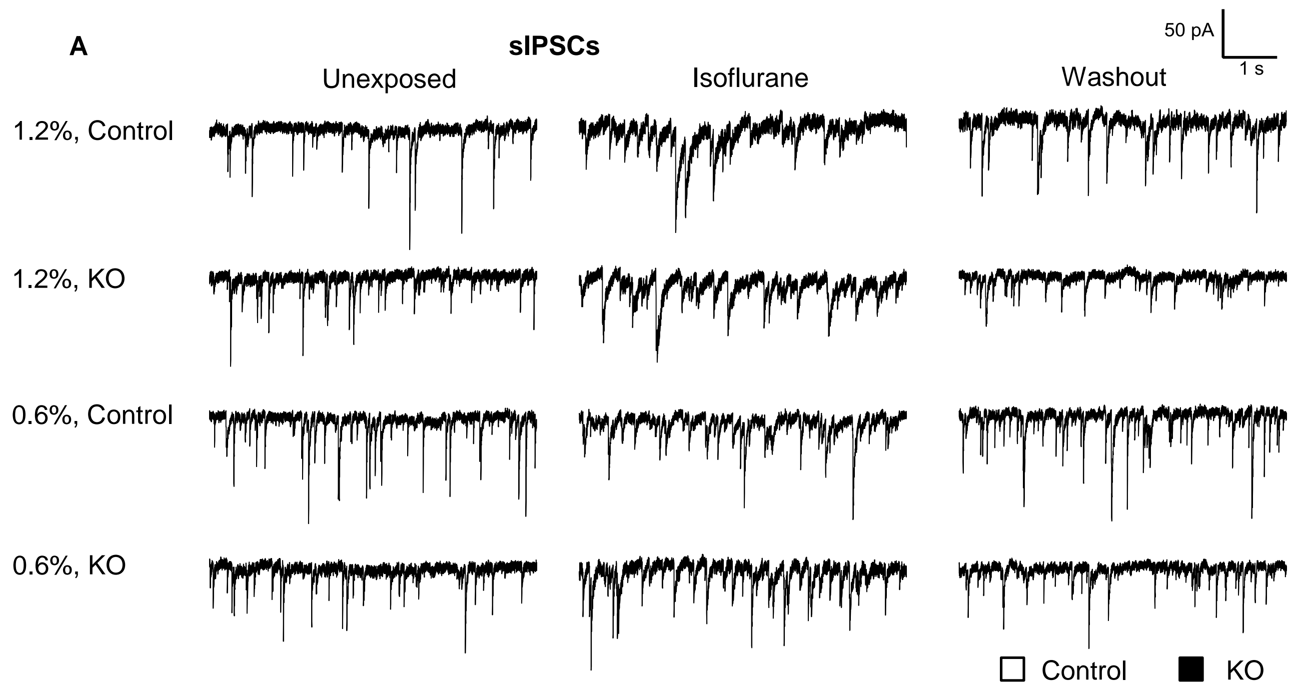
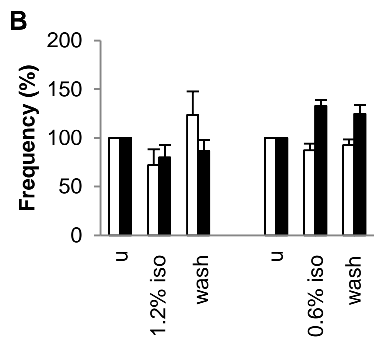
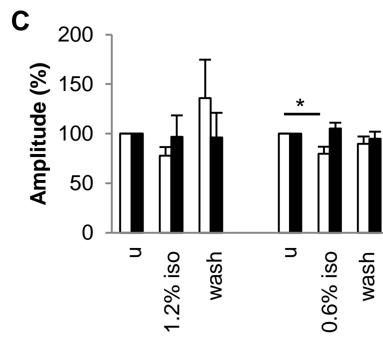


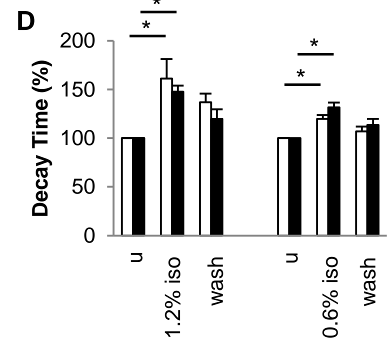
Figure S1. Effect of mitochondrial complex I dysfunction in CHAT-specific and GABA-specific neurons on sEPSC parameters in hippocampal CA1 pyramidal neurons at baseline and upon exposure to isoflurane, related to Figure 3. (**A-C**) sEPSC frequency, amplitude, and decay time in CHAT-specific control and KO slices. No statistically significant changes were observed in frequency (P = 0.888, Control: n = 11 cells, CHAT-specific KO: n= 17 cells), amplitude (P = 0.043) or decay time (P = 0.851) between CHAT-specific control and KO cells. (**D-F**) sEPSC frequency, amplitude, and decay time in GABA-specific control and KO slices. No statistically significant changes were observed in frequency (P = 0.207, Control: n = 14 cells, GABA-specific KO: n = 24 cells), amplitude (P = 0.425) or decay time (P = 0.042) between GABA-specific control and KO cells. (**G-I**) Changes in sEPSC relative frequency, relative amplitude, and relative decay time upon isoflurane exposure in CHAT-specific KO slices (Control: n = 7 cells, KO: n = 13 cells). 0.6% isoflurane exposure did not affect sEPSC frequency (Control: P = 0.112, KO: P = 0.239), amplitude (Control: P = 0.305, KO: P = 0.368) and sEPSC decay time (Control: P = 0.964, KO: P = 0.397) recorded from CHAT-specific control slices and KO slices. (**J-L**) Changes in in sEPSC relative frequency, relative amplitude, and relative decay time upon isoflurane exposure in GABA-

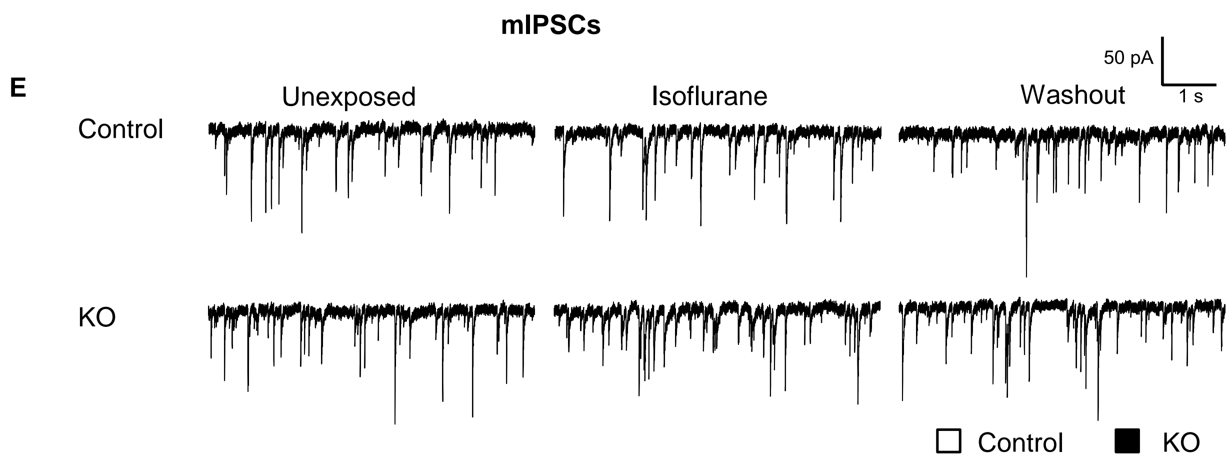
specific KO slices (Control: n = 12 cells, KO: n = 16 cells). 0.6% isoflurane exposure did not affect sEPSC frequency (Control: P = 0.018, KO: P = 0.022), amplitude (Control: P = 0.046, KO: P = 0.646) or sEPSC decay time (Control: P = 0.472, KO: P > 0.05) recorded from GABA-specific control slices and KO slices. Graph bars here and in subsequent figures represent the mean, error bars represent standard error of the mean.

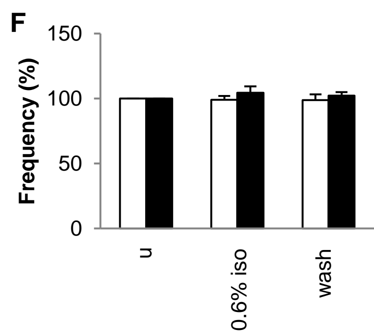

A
sIPSCs
50 pA
1 s
Unexposed
Isoflurane
Washout
1.2%, Control
1.2%, KO
0.6%, Control
0.6%, KO
Control
KO

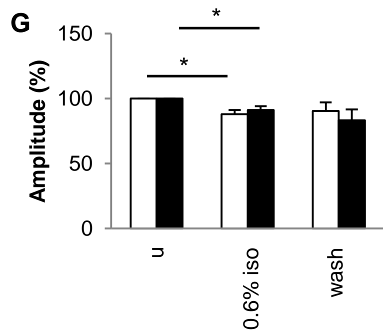

B
Frequency (%)
200
150
100
50
0
u
1.2% iso
wash
u
0.6% iso
wash


C
Amplitude (%)
200
150
100
50
0
*
u
1.2% iso
wash
u
0.6% iso
wash


D
Decay Time (%)
200
150
100
50
0
*
*
*
*
u
1.2% iso
wash
u
0.6% iso
wash


mIPSCs
E
50 pA
1 s
Unexposed
Isoflurane
Washout
Control
KO
Control
KO


F
Frequency (%)
150
100
50
0
u
0.6% iso
wash


G
Amplitude (%)
150
100
50
0
*
*
u
0.6% iso
wash

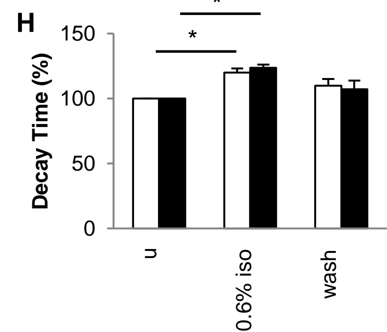

H
Decay Time (%)
150
100
50
0
*
*
u
0.6% iso
wash

Figure S2. Effect of global *Ndufs4* knock-out on sIPSC and mIPSC parameters in hippocampal CA1 pyramidal neurons upon exposure to isoflurane, related to Figure 3. (**A**) Representative sIPSC traces before isoflurane exposure (Unexposed), during isoflurane exposure (Isoflurane), and after washout (Washout). Isoflurane concentration and genotype group are shown on the left. (**B-D**) Quantification of relative sIPSC frequency, relative amplitude, and relative decay time (1.2% isoflurane: control n = 5 cells, KO n = 5 cells; 0.6% isoflurane: control n = 7 cells, KO n = 5 cells). 1.2% isoflurane exposure significantly increased sIPSC decay time obtained from both control cells ($P < 0.05$) and KO cells ($P < 0.05$). 0.6% isoflurane exposure also significantly increased sIPSC decay time obtained from both control cells ($P < 0.05$) and KO cells ($P < 0.05$). sIPSC amplitude of control cells decreased upon exposure to 0.6% isoflurane ($P < 0.05$). 1.2% isoflurane exposure did not affect sIPSC amplitude (Control: $P = 0.367$, KO: $P = 0.367$) or frequency (Control: $P = 0.182$, KO: $P = 0.367$) of both control and KO cells. 0.6% isoflurane exposure decreased sIPSC amplitude in control cells ($P < 0.05$) without affecting sIPSC amplitude in KO cells ($P = 0.093$). 0.6% isoflurane exposure did not affect sIPSC frequency in both control and KO cells (Control: $P = 0.486$, KO: $P = 0.093$). (**E**) Representative mIPSC traces before isoflurane exposure (Unexposed), during 0.6% isoflurane exposure (0.6% isoflurane), and after washout (Washout). (**F-H**) Quantification of relative mIPSC frequency, relative amplitude, and relative decay time (Control: n = 7 cells, KO: n = 7 cells). 0.6% isoflurane exposure did not affect mIPSC frequency (Control: $P = 0.964$, KO: $P = 0.486$). 0.6% isoflurane exposure decreased the amplitude of both control and KO cells (Control: $P < 0.05$, KO: $P < 0.05$), and increased decay time of both control and KO cells (Control: $P < 0.05$, KO: $P < 0.05$) of both control and KO cells.

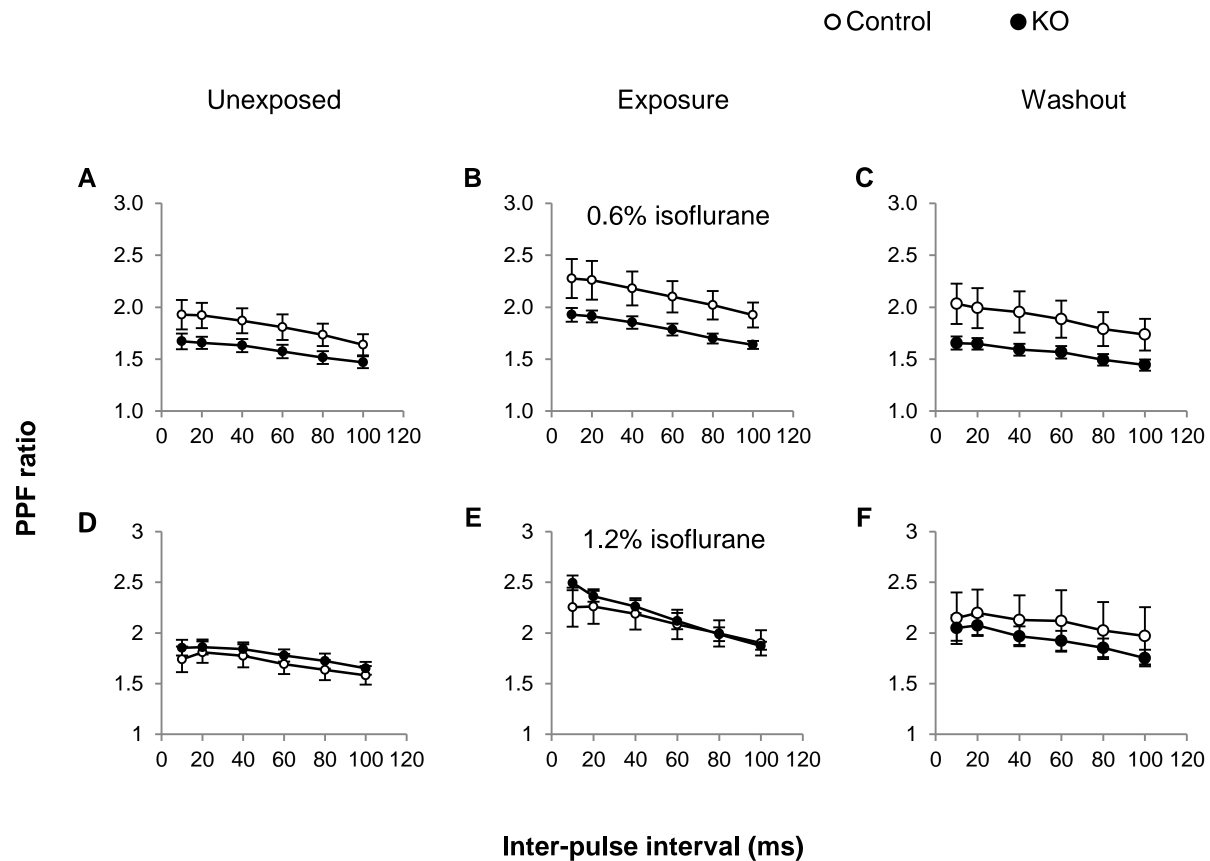


Figure S3. Inter-pulse interval profiles of PPF ratios for global KO and control slices at baseline and after isoflurane exposure, related to Figure 4. (A-C) PPF ratios at various inter-pulse intervals for 0.6% isoflurane experiment: prior to exposure, after 0.6% isoflurane exposure and after washout. (D-F) Inter-pulse interval profiles for 1.2% isoflurane experiment: prior to exposure, after 1.2% isoflurane exposure, and after washout. No significant differences between KO and control slices in PPF ratios were identified at either inter-pulse interval and at either treatment group (P > 0.05, n = 5 slices).

Supplemental Experimental Procedures

Animals

All animal experiments were performed in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee of Seattle Children's Research Institute.

Mice were maintained on a standard rodent diet with 12 hours dark-light cycle at 22°C. Water and food were available *ad libitum*. Both male and female mice were used for all experiments. Mice heterozygous for *Ndufs4* null allele (*Ndufs4*$^{\Delta/+}$) on C57BL/6 genetic background were crossed to produce wild-type (*Ndufs4*$^{+/+}$), heterozygous (*Ndufs4*$^{\Delta/+}$) and knock-out (*Ndufs4*$^{\Delta/\Delta}$, KO or total KO) animals. The offspring genotype was determined by polymerase chain reaction. KO 23-30 days-old mice were compared with the age-matched control animals. Since there is no reported difference in NDUFS4 protein expression levels [S1], in phenotype [S1, S2], in isoflurane or halothane sensitivity (data not shown) and in electrophysiological properties of CA1 pyramidal neurons between wild-type and heterozygous mice (data not shown), both genotypes were used for the control group.

GABAergic-specific *Ndufs4* KO (*Gad2*$^{Cre/+}$*::Ndufs4*$^{lox/lox}$, GABA-specific KO), VGLUT2-specific *Ndufs4* KO (*Slc17a6*$^{Cre/+}$*::Ndufs4*$^{\Delta/lox}$, VGLUT2-specific KO), and cholinergic-specific *Ndufs4* KO (*Chat*$^{Cre/+}$*::Ndufs4*$^{lox/lox}$, CHAT-specific KO) were compared to their siblings heterozygous for *Ndufs4*^{lox} and heterozygous for *Cre* driver. Mouse weight information is presented in Table S1. No animals were excluded from analysis. The offspring genotype was determined by polymerase chain reaction. Cre expression was localized to glutamatergic neurons in the hypothalamus, thalamus, midbrain, hindbrain, and spinal cord for *Slc17a6*$^{Cre/+}$ line [S3], to GABAergic neurons in all brain regions for *Gad2*$^{Cre/+}$ line [S4], and to cholinergic CNS sites including nucleus basalis, medial septum, vertical diagonal band of broca, laterodorsal tegmental area, peduncular pontine tegmental nucleus, nucleus ambiguous, and cranial nerve nuclei for *Chat*$^{Cre/+}$ line [S5]. Each one of the founder lines (*Ndufs4*$^{lox/lox}$, *Ndufs4*$^{\Delta/+}$, and the different Cre lines) had been backcrossed more than 10 times. The breeding strategy is *Cre/+::Ndufs4*$^{\Delta/+}$ crossed to an *Ndufs4*$^{lox/lox}$ or *Cre/+::Ndufs4*$^{lox/lox}$ crossed to an *Ndufs4*$^{lox/lox}$ mice, depending on the robustness of the Cre line and to minimize ectopic recombination. Cell-specific KO progeny did not contain any wild-type *Ndufs4* allele in any case. Animals are all genotyped using tail DNA and tested for absence of ectopic recombination at the end of the experiment using brain DNA.

Slices from GABAergic-specific *Ndufs4* KO (*Gad2*$^{Cre/+}$*::Ndufs4*$^{lox/lox}$, GABA-specific KO) and cholinergic-specific *Ndufs4* KO (*Chat*$^{Cre/+}$*::Ndufs4*$^{lox/lox}$, CHAT-specific KO) were compared to slices from their siblings heterozygous for *Ndufs4*^{lox}.

Anesthetic sensitivity

Mice were anesthetized by gradually increasing isoflurane or halothane concentration while animal body temperature was maintained by radiant heat. Responses of the same mouse to different concentration of the same anesthetic were determined after 15 min of equilibration between different anesthetic concentrations. The EC_{50} for failure to respond to non-damaging tail clamp was determined by the bracketing method [S6]. Isoflurane and halothane were sampled from the exposure chamber and their concentrations were determined by gas chromatography.

Preparation of hippocampal slices

Mice were anesthetized with isoflurane, their brains were quickly removed and sliced (350 µm thick coronal plane) using the Leica VT1000S vibratome in an ice-cold oxygenated (95% O_2, 5% CO_2, carbogen) slicing solution (in mM: KCl 5, NaH_2PO_4 1.25, $MgSO_4$ 3.5, $CaCl_2$ 0.5, $NaHCO_3$ 26, glucose 10, sucrose 210). After incubation in oxygenated slicing solution for 30 min at room temperature, slices were transferred to a chamber containing oxygenated artificial cerebrospinal fluid (ACSF, in mM: NaCl 118, KCl 3, $CaCl_2$ 1.5, $MgCl_2$ 1, $NaHCO_3$ 25, NaH_2PO_4 1, glucose 30). Slices were allowed to equilibrate for at least 30 min in ACSF at room temperature. Individual slices were transferred to the recording chamber, which was superfused with oxygenated ACSF at 3.2-3.8 ml/min flow rate. Due to difficulty in maintaining gigaohm seals for the duration of isoflurane exposure experiment at 37°C, all experiments on slices were performed at 30°C. Isoflurane was applied in the superfusate at equilibrated concentrations delivered by passing carbogen through a calibrated isoflurane vaporizer (Scivena Scientific TEC-3

and Patterson Veterinary TEC-3). The superfusate was sampled during isoflurane exposure, and the isoflurane concentration was determined using gas chromatography.

Whole-cell patch-clamp experiments

Hippocampal pyramidal cells were visualized using differential interference contrast microscopy. Whole-cell patch-clamp experiments were performed using borosilicate glass capillaries pulled on Sutter Instruments P-97 puller. Patch pipettes were filled with either a potassium gluconate-based solution (in mM: potassium gluconate 140, $CaCl_2$ 1, $MgSO_4$ 2, EGTA 10, Na_2ATP 4, NaGTP 0.4, HEPES 10) for recording spontaneous excitatory post-synaptic currents (sEPSCs) and miniature excitatory post-synaptic currents (mEPSCs) or a cesium chloride-based solution (in mM: CsCl 140, HEPES 10, phosphocreatine 10, Mg-ATP 4, Na-GTP 0.3, QX314-Br 5) for recording spontaneous inhibitory post-synaptic currents (sIPSCs) and miniature inhibitory post-synaptic currents (mIPSCs). Internal solution pH was adjusted to 7.3 using potassium hydroxide or cesium hydroxide respectively, final osmolality was 309-314 mOsm. In addition, 10 μM α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonist 6,7-Dinitroquinoxaline-2,3-dione disodium (DNQX) and 10 μM *N*-Methyl-D-aspartate (NMDA) antagonist (*RS*)-3-(2-Carboxypiperazin-4-yl)-propyl-1-phosphonic acid ((RS)-CPP) were included in the external solution for recording sIPSCs. For recording mEPSCs and mIPSCs, 0.5 μM tetrodotoxin (TTX) was applied to the superfusate. Patch pipettes had a resistance of 3 to 5 MΩ. Recordings were performed with either an EPC10 double amplifier (HEKA) and digitized with a Digidata 1322A (Axon Instruments) or a MultiClamp700B Amplifier (Axon Instruments) and digitized with a Digidata 1400 (Axon Instruments). Patch-clamp currents were filtered at 2.1 kHz and sampled at 10 kHz. Holding potential was -60 mV. Series resistance was monitored during the experiments; recordings with substantially changed series resistance were excluded from the analysis.

Extracellular experiments

Extracellular recording electrodes were fabricated by pulling borosilicate glass capillaries on Sutter Instruments P-97 puller, breaking the tip against sand paper, and filling with ACSF. Pipette resistance was less than 1 MΩ. The bipolar stimulating electrode was fabricated by twisting 2 Teflon-coated platinum wires (50 μm, AM-Systems). Stimuli were delivered by A-365 stimulus isolation unit (WPI). The stimulating electrode was positioned on Schaffer collateral fibers, while recording electrode was positioned in CA1 *stratum radiatum* for recording extracellular field excitatory post-synaptic potentials (fEPSPs). The amplitude of stimulation ranged from 50 to 150 μA, and was selected to produce half-maximal fEPSP amplitude. The stimulus duration was 100 μs. Field potential signals were amplified using EPC10 amplifier (HEKA) and digitized with Digidata 1322A (Axon Instruments) at 200 kHz sampling rate. Two-pulse protocol was used with 60 ms inter-pulse interval for isoflurane time course experiments. In some cases inter-pulse intervals in the range of 10 to 100 ms were used before isoflurane exposure, during isoflurane exposure and after isoflurane wash. Stimulations were applied at 0.033 Hz (every 30 s).

Data Analysis

Traces were analyzed with pClamp 10 software (Axon Instruments). sEPSCs, mEPSCs, sIPSCs and mIPSCs were analyzed for amplitude, frequency, and 90%-10% decay time. Due to high variability of baseline parameters, exposed and wash data were normalized to their average unexposed parameter and expressed as % of unexposed value. Extracellular traces were digitally filtered off-line at 10 kHz, and fEPSP amplitudes were analyzed using pClamp software (Axon). fEPSP amplitudes were normalized as the percent of the average of fEPSP amplitudes during 10 minutes of recording immediately preceding isoflurane exposure. Paired-pulse facilitation (PPF) ratios were calculated by dividing the amplitude of the second peak to the amplitude of the first peak. Data are expressed and graphed as means ± standard errors of the mean of measured responses. Cell specific EC_{50}s are expressed as means ± standard deviations. Statistical analyses (two way ANOVA with post Tukey test for cell-specific *in vivo* data, Mann-Whitney *U* test for 2 group non-paired comparisons, Friedman test for repeated measures comparisons with post Tukey test) were performed in SigmaPlot 12.5. For comparison of controls and total KO values, significance was taken as $p<0.05$. When comparing changes in parameters for synaptic functions of EPSCs for exposure to 0.6% isoflurane, we compared six groups (3 different control groups, global KO, 2 cell specific KOs). Therefore, we used a Bonferroni correction for six groups leading to a level of 0.01 for significance.

References

S1. Kruse, S.E., Watt, W.C., Marcinek, D.J., Kapur, R.P., Schenkman, K.A., and Palmiter, R.D. (2008). Mice with mitochondrial complex I deficiency develop a fatal encephalomyopathy. Cell Metab *7*, 312-320.

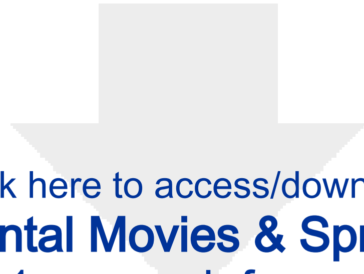
S2. Quintana, A., Kruse, S.E., Kapur, R.P., Sanz, E., and Palmiter, R.D. (2010). Complex I deficiency due to loss of Ndufs4 in the brain results in progressive encephalopathy resembling Leigh syndrome. Proc. Natl. Acad. Sci. U. S. A. *107*, 10996-11001.

S3. Borgius, L., Restrepo, C.E., Leao, R.N., Saleh, N., and Kiehn, O. (2010). A transgenic mouse line for molecular genetic analysis of excitatory glutamatergic neurons. Mol. Cell. Neurosci. *45*, 245-257.

S4. Taniguchi, H., He, M., Wu, P., Kim, S., Paik, R., Sugino, K., Kvitsiani, D., Fu, Y., Lu, J., Lin, Y., et al. (2011). A resource of Cre driver lines for genetic targeting of GABAergic neurons in cerebral cortex. Neuron *71*, 995-1013.

S5. Rossi, J., Balthasar, N., Olson, D., Scott, M., Berglund, E., Lee, C.E., Choi, M.J., Lauzon, D., Lowell, B.B., and Elmquist, J.K. (2011). Melanocortin-4 receptors expressed by cholinergic neurons regulate energy balance and glucose homeostasis. Cell Metab *13*, 195-204.

S6. Sonner, J.M., Gong, D., Li, J., Eger, E.I., 2nd, and Laster, M.J. (1999). Mouse strain modestly influences minimum alveolar anesthetic concentration and convulsivity of inhaled compounds. Anesth. Analg. *89*, 1030-1034.



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