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Halogenated volatile anesthetics alter brain metabolism as revealed by proton magnetic resonance spectroscopy of mice *in vivo*

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ABSTRACT

Halogenated volatile anesthetics (HVA) are widely used in medicine and research but their effects on brain metabolism in intact organisms are still largely unknown. Here, localized proton magnetic resonance spectroscopy (MRS) of anesthetized mice was applied to evaluate HVA effects on cerebral metabolites *in vivo*. Experimental protocols combined different concentrations of isoflurane, halothane, sevoflurane, and desflurane with known modulators of adrenergic, GABAergic, and glutamatergic neurotransmission. As a most striking finding, brain lactate increased in individual mice from 1.0 ± 0.6 mM (awake state) to 6.2 ± 1.5 mM (1.75% isoflurane). In addition, relative to total creatine, there were significant isoflurane-induced increases of alanine by 111%, GABA by 20%, choline-containing compounds by 20%, and *myo*-inositol by 10% which were accompanied by significant decreases of glucose by 51% and phosphocreatine by 9%. The elevation of lactate was most pronounced in the striatum. The HVA effects correlated with the respective minimal alveolar concentrations and were mostly reversible within minutes. The observed alterations are best explained by an HVA-induced stimulation of adrenergic pathways in conjunction with an inhibition of the respiratory chain. Apart from casting new light on cerebral energy metabolism, the present results challenge brain studies of HVA-anesthetized animals.

Introduction

General anesthesia was introduced in 1847 with the halogenated solvent chloroform. Modern halogenated volatile anesthetics (HVA) like isoflurane or sevoflurane are frequently used in general anesthesia due to their high potency, rapid reversible action and reasonable benefit-to-risk ratio (Miller, 2000). So far, possible mechanisms of action have mostly been investigated in cell homogenates, cell cultures, and brain slices and include relatively unspecific alterations of membrane fluidity and conformation of membrane-bound proteins that lead to a broad range of effects (Miller, 2000). More specific actions of HVA refer to the modulation of GABAergic (Hall et al., 1994; Mihic et al., 1997; Westphalen and Hemmings, 2006), glutamatergic (Bickler et al., 1994; Lee et al., 2009; Westphalen and Hemmings, 2006), and cholinergic pathways (Gomez et al., 2000; Griffiths et al., 1994; Xu et al., 2000).

HVA like isoflurane also affect the energy metabolism and possibly block respiratory chain complex I (Nahrwold and Cohen, 1973). Studies of neuronal cell cultures and in mice *post mortem* suggest an activation of the mitochondrial apoptotic pathway (Zhang et al., 2011). Earlier work in monkey kidney cells indicated a concentration-dependent increase of lactate under isoflurane (Brabec et al., 1984). On the other hand, there is only sparse data on the influence of HVA on the intact brain of living animals or humans. Recently, an increase of extracellular brain lactate was reported in mice *in vivo* by microdialysis (Horn and Klein, 2010), while a higher lactate level was found in peripheral blood under isoflurane compared to thiopental (Duarte and Gruetter, 2012). Moreover, isoflurane has been shown to cause cerebral vasodilation and to reduce cerebral metabolic consumption of oxygen and glucose (Alkire et al., 1997; Toyama et al., 2004). A recent study reported developmental neurotoxicity after 6 hours of 1.5% isoflurane exposure (Loepke et al., 2009).

Localized proton magnetic resonance spectroscopy (MRS) offers the unique opportunity of monitoring cerebral metabolism in living animals or humans. Here, we employed *in vivo* MRS of mice to directly assess metabolic changes in response to HVA anesthesia. In order to

Abbreviations: ADP, adenosine diphosphate; Ala, alanine; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; Cho, choline-containing compounds; CNS, central nervous system; CSF, cerebrospinal fluid; FDG, fluorodesoxyglucose; GABA, γ-aminobutyric acid; Gln, Glutamine; Glu, Glutamate; HVA, halogenated volatile anesthetics; Ins, *myo*-inositol; MAC, minimal alveolar concentration; MRS, localized proton magnetic resonance spectroscopy; NAA, *N*-acetylaspartate; PDH, pyruvate dehydrogenase; PI, phosphatidylinositol; Tau, taurine; TCA, tricarboxylic acid cycle; tCr, total creatine (sum of PCr: phosphocreatine and Cr: creatine).

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differentiate underlying mechanisms of action, we compared various anesthetic agents at different concentrations as well as with and without modulators of adrenergic, GABAergic, and glutamatergic neurotransmission.

Materials and methods

Animals and study design

All studies were performed in accordance with German animal protection laws after approval by the responsible governmental authority (Az 33.14-42502-04-102/09). Unless stated otherwise, all mice (healthy, female, adult, NMRI, total number = 78) were initially anesthetized with 5% isoflurane, subsequently intubated, positioned on the Göttingen animal bed (Boretius et al., 2009) and kept under anesthesia with 1.75% isoflurane in ambient air. Subsequently, different anesthesia protocols were applied as described below. In all experiments mice were mechanically ventilated at intermittent positive pressure with a constant respiration frequency of 85 breaths per minute (*Animal Respirator Advanced*™, TSE Systems, Germany) to ensure stable and well-defined isoflurane and oxygen supply. Body temperature was kept constant at 36.0 ± 0.5 °C. To reduce acoustic stress in periods without anesthetics, the ears of the mice were plugged by selfmade hearing protectors. Moreover, the room was kept dark.

MRS of regional brain metabolites

In vivo localized proton MRS (STEAM, outer volume suppression) was performed under fully relaxed conditions (TR/TE/TM = 6000/10/10 ms) in different brain regions of mice at a field strength of 9.4 T (Bruker BioSpin MRI GmbH, Ettlingen, Germany). Shimming was carried out by FASTMAP (Gruetter, 1993). For proper positioning of volumes-of-interest (VOI) T2-weighted images (2D FSE, TR/TE = 4200/43 ms, 8 echoes, resolution 100 × 100 μm^2, slice thickness 500 μm) were obtained in axial and sagittal orientation. Apart from regional studies (see below), serial spectra of the cerebrum (number of averages NA = 32) were obtained every 5 min leaving enough time to adjust, if necessary, the resonance frequency and water suppression in between acquisitions. Metabolite quantification involved spectral evaluation by LCModel (Version 6.2-0, Provencher, 1993) and calibration with a brain water concentration of 43.7 mol/l (Schwarcz et al., 2003). Values with Cramer–Rao lower bounds above 20% were excluded from further analyses unless noted otherwise.

To assess regional differences, spectra were obtained in 5 different brain regions of 12 mice in random order under 1.75% isoflurane: mid-frontal cortex (3.9 × 0.7 × 3.2 mm^3, NA = 64–256, n = 12), left hippocampus (1.8 × 1.2 × 1.8 mm^3, NA = 128–256, n = 9), left striatum (1.2 × 1.4 × 2.0 mm^3, NA = 256, n = 8), CSF (3.3 × 2.2 × 1.8 mm^3, NA = 64–128, n = 6), and cerebrum (4 × 3 × 4 mm^3, NA = 32, n = 7).

Modulation of brain metabolism by isoflurane

After measuring 5 spectra of the cerebrum at 1.75% isoflurane, the anesthetic was switched off (0%), while continuously acquiring spectra every 5 min (n = 22). After 35 min, isoflurane was switched back to 1.75% (n = 6). To avoid movement artifacts in periods without isoflurane, the neuromuscular blocking agent pancuronium bromide (0.15 mg/kg s.c.) was administrated 15 min before switching off the isoflurane supply.

Glucose infusion, oxygen supply, and hypercapnia

After reference MRS at 1.75% isoflurane, 130 mg/kg glucose (1.075 M) was administrated as a bolus injection via a tail vein catheter followed by a constant glucose infusion (n = 1, 0.97 g/h/kg) over 125 min. Spectra of the cerebrum were obtained for 270 min under constant isoflurane at 1.75%.

To rule out systemic hypoxia as a cause for metabolic changes, 6 animals received either ambient air (n = 3) or ambient air + 50% oxygen (n = 3) as carrier gas. MRS of the cerebrum was performed 30 min after starting anesthesia (3 spectra). After one week these measurements were repeated using the other carrier gas, respectively.

Following the standard reference period, MRS was performed after adding 30% CO_2 while keeping the total gas flow constant (n = 1). After 25 min the CO_2 addition was reduced to 20% and after additional 25 min to 10%. Twenty-five minutes later, the CO_2 addition was switched off.

Pharmacologic modulation of isoflurane-induced metabolite profiles

After the acquisition of baseline spectra at 1.75% isoflurane, one of the following drugs was administered as a single injection dose: medetomidine (n = 4, 0.5 mg/kg s.c.), atipamezole (n = 4, 2.5 mg/kg s.c.), clonidine (n = 3, 5 mg/kg i.p.), propranolol (n = 3, 20 mg/kg i.p.), ketamine (n = 4, 150 mg/kg s.c.), dichloroacetate (n = 3, 50 mg/kg i.p.), diazepam (n = 3, 50 mg/kg i.p.), and pentobarbital-sodium (n = 2, 45 mg/kg i.p.). Spectra were obtained for typically 100 min under constant isoflurane supply (1.75%) with exception of pentobarbital, where isoflurane was switched off 30 min after drug delivery. In addition, the medetomidine group received atipamezole (2.5 mg/kg s.c.) and spectra were obtained for further 60 min.

Different HVA

To investigate the neurochemical profile induced by HVA other than isoflurane, mice were initially anesthetized with 4% halothane, 6% sevoflurane, or 12% desflurane, respectively. The animals were then intubated, positioned on the Göttingen animal bed, and kept under anesthesia with 1.25% halothane, 3% sevoflurane, or 9% desflurane. Subsequently, different anesthetic protocols were applied including series with increasing HVA concentrations and intermediate periods without HVA (see Results).

In addition, for each HVA, two mice were initially anesthetized with 5% isoflurane. After the acquisition of baseline spectra at 1.75% isoflurane, the anesthetic was switched off. Spectra were continuously acquired for 35 min before the respective HVA was given.

To avoid movement artifacts in periods without HVA, the neuromuscular blocking agent pancuronium bromide (0.15 mg/kg s.c.) was administrated 15 min before switching off the isoflurane supply and, in prolonged experiments, once again after about one hour (0.075 mg/kg s.c.).

Non-volatile anesthetics

Following baseline MRS and 35 min after withdrawal of 1.75% isoflurane, mice received either ketamine/medetomidine (n = 2, 150/0.25 mg/kg s.c.), medetomidine (n = 3, 0.5 mg/kg s.c.), pentobarbital (n = 2, 45 mg/kg i.p.), diazepam (n = 1, 25 mg/kg i.p.) or fentanyl-citrate (n = 1, 0.5 mg/kg i.p.) and additional spectra were obtained over 40–45 min.

Controls

After baseline MRS mice were kept under constant isoflurane (1.75%) either without further medication (n = 5) or after receiving physiological saline (n = 6, 0.15–0.20 ml, 0.9% NaCl i.p.) or pancuronium bromide (n = 2, 0.15 mg/kg i.p.). Because only minor differences between these treatments were observed, the data of the three control groups were pooled (Supplementary Fig. 1).

Pharmacokinetic–pharmacodynamic modeling

To further understand the relation between the HVA concentration in breathing air and the resulting brain lactate concentration, spectra were obtained at different HVA concentrations in at least 3 different animals using a variety of different administration protocols (see Results). Brain lactate concentrations were analyzed as a function of the nature and concentration of the breathing gas by pharmacokinetic nonlinear mixed-effects modeling with NONMEM version VI (GloboMax, Hanover, MD, USA) using the ADVAN6 subroutine (model specification using non-stiff differential equations) and the first-order conditional estimates method for minimization. In total, 43 different experiments with 1245 lactate data points and at least 3 different animals per HVA were included in nonlinear mixed-effects modeling. According to both the objective function and visual inspection for systematic deviations between predicted and observed values, brain lactate concentrations were best described by an effect compartment model (Sheiner et al., 1979) describing the HVA transition into the brain (Eq. (1)) and an indirect response model (Dayneka et al., 1993) describing the HVA-related inhibition of cerebral lactate elimination (Eq. (2)). With k_{e0} as transfer constant between the HVA in the vaporizer and brain tissue, Eqs. (1) and (2) read

$$d[HVA_{Brain}]/dt = k_{e0} * \left(\left[HVA_{Vaporizer}\right] - [HVA_{Brain}]\right) \quad (1)$$

and

$$d[Lactate_{Brain}]/dt = [HVA_{Brain}] * f_m + [Lactate]_0 - k_e * (1-(I_{max} * [HVA_{Brain}]/(IC_{50} + [HVA_{Brain}])) * [Lactate_{Brain}], \quad (2)$$

respectively, using f_m as baseline lactate formation rate, $[Lactate]_0$ as baseline cerebral lactate, $[Lactate]_{Brain}$ as measured cerebral lactate, k_e as baseline lactate elimination rate, I_{max} as maximal inhibition constant for lactate elimination and IC_{50} as the HVA concentration resulting in half-maximum inhibition of lactate elimination.

Statistics

All statistical analyses were performed with SPSS (IBM, USA) version 16. Data were described by mean and standard deviation for experimental results and by standard error of the mean when discussing the effects of a procedure. Multiple group comparisons such as comparisons between different brain regions were performed using one-way ANOVA with Bonferroni *post hoc* test when adequate. Considering the fact that the main focus of the study was on the evaluation of isoflurane-induced alterations of 12 metabolites, an appropriately adjusted level of significance would be 0.0042. No adjustments were required in additional experiments designed to address specific questions such as the inhibition of HVA effects by antiadrenergic drugs. For intraindividual comparisons paired *t*-tests were performed.

Results

Modulation of brain metabolism by isoflurane

Isoflurane anesthesia of female NMRI mice at a common concentration of 1.75% resulted in a rapid and pronounced increase of brain lactate and alanine that was accompanied by moderate changes in glucose, creatine, glutamate, GABA, choline-containing compounds, and *myo*-inositol. Table 1 quantifies individual changes between isoflurane anesthesia and an awake state 20 to 25 min after terminating isoflurane. In particular, isoflurane-induced levels of 6.2 ± 1.5 mM lactate and 1.6 ± 0.4 mM alanine decreased to 1.0 ± 0.6 mM and 0.7 ± 0.4 mM, respectively.

Table 1
Isoflurane-induced changes of absolute metabolite concentrations in mouse brain *in vivo* ($n = 22$).

Metabolites	n[a]	Without isoflurane	With 1.75% isoflurane[b]	Mean paired difference	p[c]
Lactate	7	1.2 ± 0.8	7.2 ± 1.3	6.0 ± 1.0	0.000005
	17[d]	1.0 ± 0.6	6.2 ± 1.5	5.3 ± 1.0	<0.000001
Alanine	4	1.1 ± 0.6	1.9 ± 0.3	1.0 ± 0.5	0.028
	16[d]	0.7 ± 0.4	1.6 ± 0.4	0.9 ± 0.3	<0.000001
Glucose	12	4.5 ± 0.5	2.3 ± 1.0	−2.2 ± 1.3	<0.001
N-Acetylaspartate	22	6.2 ± 0.4	6.5 ± 0.5	0.3 ± 0.3	0.001
Total Creatine	22	7.9 ± 0.4	8.2 ± 0.5	0.3 ± 0.5	0.003
Phosphocreatine	22	2.7 ± 0.3	2.6 ± 0.3	−0.2 ± 0.4	0.062
Creatine	22	5.2 ± 0.3	5.7 ± 0.4	0.5 ± 0.3	<0.001
Choline-containing compounds	22	2.0 ± 0.2	2.4 ± 0.2	0.5 ± 0.1	<0.001
myo-Inositol	22	3.4 ± 0.3	3.8 ± 0.3	0.4 ± 0.3	<0.001
Glutamate	22	7.9 ± 0.9	8.6 ± 0.9	0.6 ± 0.6	<0.001
Glutamine	22	3.8 ± 0.4	3.6 ± 0.4	0.2 ± 0.3	0.005
GABA	21	1.6 ± 0.3	1.9 ± 0.3	0.4 ± 0.2	<0.001

[a] Values refer to spectra with Cramer–Rao lower bounds < 20%.
[b] Metabolite concentrations (mM, mean ± standard deviation) are obtained from spectra 10 and 5 min before withdrawal of isoflurane in comparison to those obtained 20 and 25 min after isoflurane withdrawal.
[c] Paired *t* test (considering that 12 parameters were analyzed, the most conservative margin of significance would be 0.0042).
[d] Values refer to spectra with Cramer–Rao lower bounds < 60%. This choice includes very low concentrations of lactate and alanine and leads to a more reliable estimation of respective baseline concentrations.

Although movement artefacts during isoflurane-free periods were reduced by the muscle relaxant pancuronium, the MRS data revealed a mild signal decrease as indicated by the temporal profiles of total creatine and *N*-acetylaspartate (Supplementary Fig. 2). To account for the corresponding underestimation of absolute metabolite concentrations in the awake state, concentrations are also presented as ratios to total creatine (Table 2, Fig. 1). The procedure results in a constant level for NAA/tCr (Supplementary Fig. 2). Under isoflurane the ratios of lactate and alanine increased by 533% and 111%, whereas glucose and phosphocreatine ratios decreased by 51% and 9%, respectively. Other metabolite alterations included small but statistically significant increases of creatine (5%), GABA (20%), choline-containing compounds (20%), *myo*-inositol (10%), and glutamate (4%).

Table 2
Isoflurane-induced changes of metabolite concentrations relative to total creatine in mouse brain *in vivo* ($n = 22$).

Metabolite ratios	n[a]	Without isoflurane	With 1.75% isoflurane[b]	Mean paired difference	P[c]
Lactate	7	0.15 ± 0.10	0.85 ± 0.15	0.70 ± 0.12	0.000005
	17[d]	0.12 ± 0.07	0.76 ± 0.18	0.64 ± 0.15	<0.000001
Alanine	4	0.14 ± 0.07	0.23 ± 0.01	0.09 ± 0.06	0.06
	16[d]	0.09 ± 0.05	0.19 ± 0.04	0.10 ± 0.04	<0.000001
Glucose	12	0.57 ± 0.02	0.28 ± 0.12	−0.29 ± 0.14	0.028
N-Acetylaspartate	22	0.78 ± 0.05	0.79 ± 0.04	0.00 ± 0.03	0.6
Phosphocreatine	22	0.34 ± 0.03	0.31 ± 0.03	−0.03 ± 0.03	0.0002
Creatine	22	0.66 ± 0.03	0.69 ± 0.03	0.03 ± 0.03	0.0002
Choline-containing compounds	22	0.25 ± 0.02	0.30 ± 0.02	0.05 ± 0.02	<0.000001
myo-Inositol	22	0.42 ± 0.04	0.46 ± 0.04	0.04 ± 0.04	0.001
Glutamate	22	1.00 ± 0.10	1.04 ± 0.10	0.04 ± 0.07	0.028
Glutamine	22	0.45 ± 0.51	0.46 ± 0.46	0.01 ± 0.04	0.3
GABA	21	0.20 ± 0.04	0.23 ± 0.04	0.04 ± 0.03	0.00006

[a] Values refer to spectra with Cramer–Rao lower bounds < 20%.
[b] Metabolite concentrations relative to total creatine (mean ± standard deviation) are obtained from spectra 10 and 5 min before withdrawal of isoflurane in comparison to those obtained 20 and 25 min after isoflurane withdrawal.
[c] Paired *t* test (considering that 12 parameters were analyzed, the most conservative margin of significance would be 0.0042).
[d] Values refer to spectra with Cramer–Rao lower bounds < 60%.

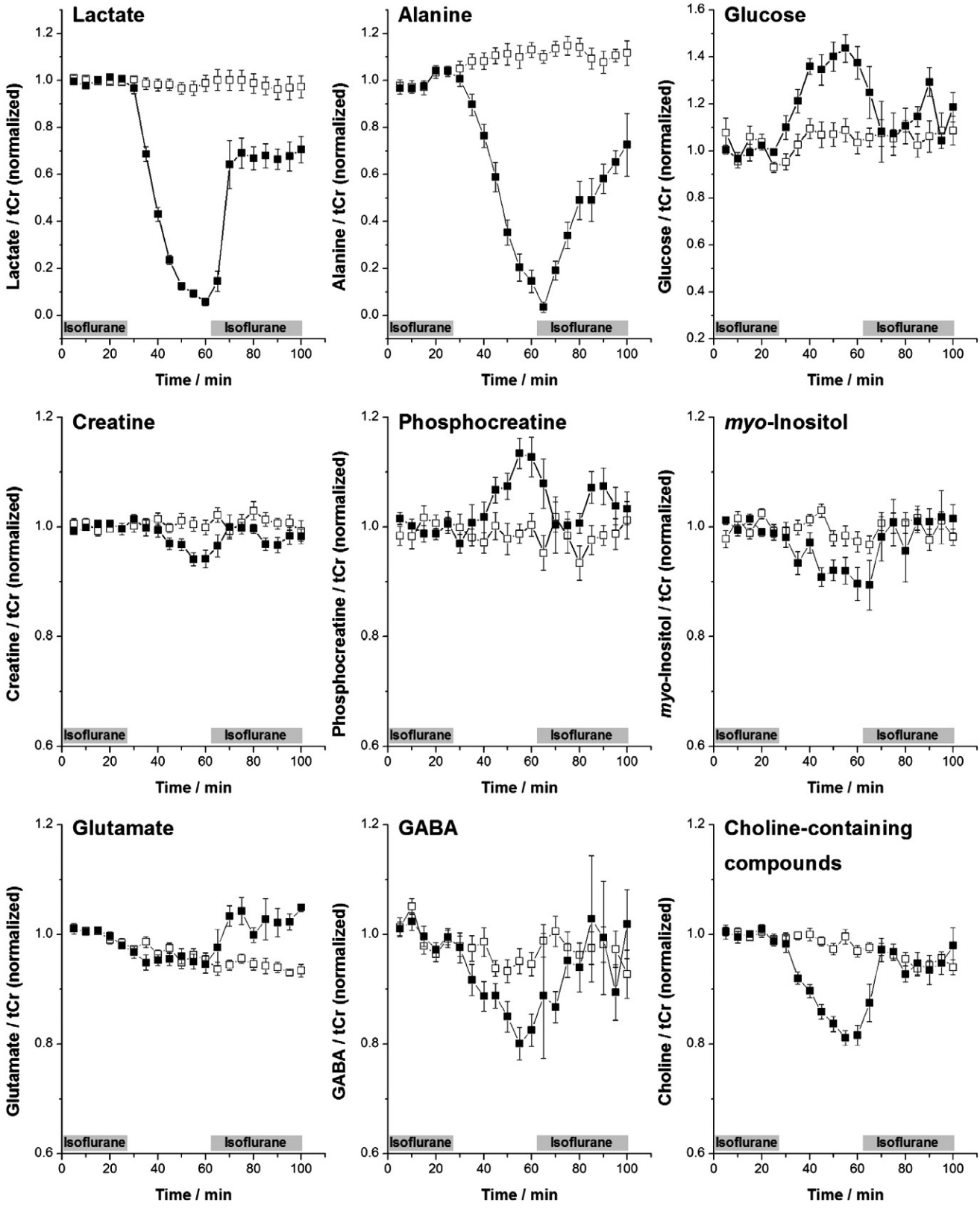

Fig. 1. Effects of isoflurane on cerebral metabolism. Relative metabolite concentrations (normalized to baseline and total creatine (tCr), mean ± standard error) as a function of time for (open symbols) mice with constant supply of 1.75% isoflurane ($n = 11$) and (solid symbols) mice subjected to a protocol with an intermediate 35 min period at 0% isoflurane ($n = 22$). Withdrawal of isoflurane led to a significant reduction of lactate and alanine as well as decreases of creatine, γ-aminobutyric acid (GABA), choline-containing compounds (Cho) and *myo*-inositol, while glucose and phosphocreatine increased significantly. The majority of changes in the awake state was reversible by re-exposure to isoflurane. For details see text.

The metabolic changes after termination of isoflurane were largely reversible within minutes when switching back to isoflurane anesthesia (Fig. 1). While lactate and alanine only partly regained the initial isoflurane-induced level, other metabolites entirely reached previous concentrations within an observation period of 35 min. Notably, the glutamate to total creatine ratio slightly decreased over

time — even under isoflurane, but increased above baseline in response to a second isoflurane exposure (Fig. 1).

Regional cerebral differences

We compared lactate concentrations in the cerebral cortex, hippocampus, and striatum (strictly avoiding CSF contamination for each volume) as well as within a larger brain volume and a volume with CSF (Fig. 2A). While isoflurane-induced increases of lactate were seen in all parts of the brain tested (Figs. 2B, C), the effect was strongest in the striatum and smallest in cerebral cortex. The striatum also presented with the highest concentrations of alanine, choline-containing compounds, and GABA under isoflurane (Supplementary Fig. 4). No regional differences were found for glucose and phosphocreatine.

Influence of glucose and oxygen supply

To further understand the effects of isoflurane on cerebral energy metabolism, we applied glucose intravenously. The procedure resulted in an increase of brain glucose from 3.5 ± 0.3 mM (5 baseline spectra) to 5.8 ± 0.3 mM (5 spectra 105–125 min after the start of infusion). However, the isoflurane-induced lactate concentration (5.3 ± 0.1 mM) was similar to that without glucose infusion (4.9 ± 0.2 mM, Supplementary Fig. 5).

To test the effect of oxygen supply, spectra were acquired both at ambient air (21% oxygen) and oxygen-enriched air (50% oxygen) in a random order. No significant differences in lactate concentrations at ambient air (5.1 ± 0.3 mM) and 50% oxygen (5.0 ± 0.5 mM) were observed (Supplementary Fig. 6). This finding suggests that a lack of

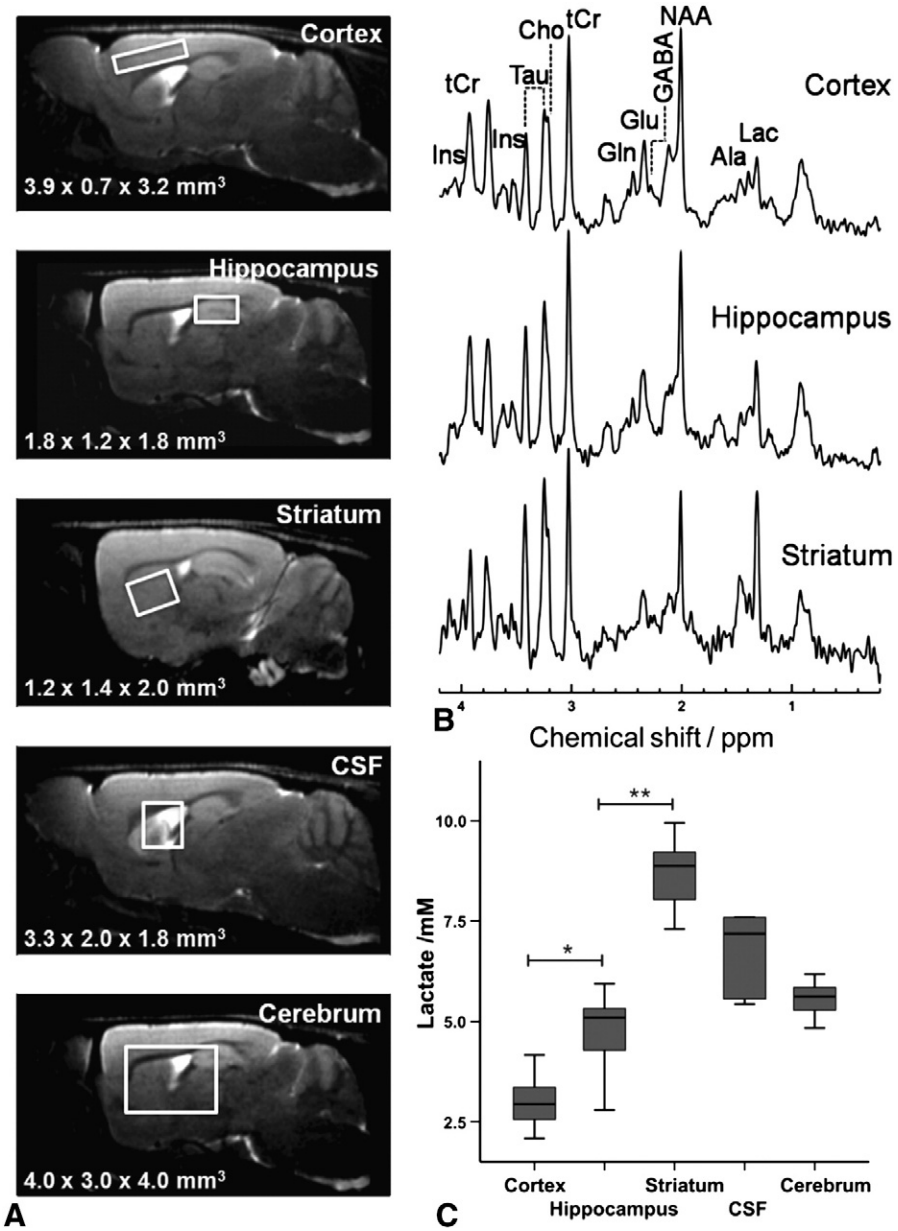

Fig. 2. (A) Volumes selected for MRS (white boxes on T2-weighted images): cerebral cortex ($n = 12$), hippocampus ($n = 9$), striatum ($n = 7$) as well as a volume of which at least 50% was cerebrospinal fluid (CSF, $n = 6$) and a larger brain volume (cerebrum, $n = 7$). (B) *In vivo* localized proton magnetic resonance spectra. (C) Isoflurane-induced lactate concentrations (median values with interquartile ranges and total range) were highest in striatum and lowest in cortex. Ala = alanine, Cho = choline-containing compounds, GABA = γ-aminobutyric acid, Gln = glutamine, Glu = glutamate, Ins = *myo*-inositol, Lac = lactate, NAA = N-acetylaspartate, Tau = Taurine, $^{*}p < 0.05$, $^{**}p < 0.000001$ (one way ANOVA, Bonferroni adjusted *post hoc t*-test).

adequate oxygen (i.e., systemic hypoxia) is not the cause for elevated lactate under 1.75% isoflurane anesthesia in ambient air. Moreover, constant isoflurane over a period of more than 2 hours did not show significant changes of lactate (Supplementary Fig. 1). The effects were also not related to the applied muscle relaxant used to avoid movement artifacts in periods without anesthesia. It is well known that pancuronium does not pass the intact blood brain barrier and thus should not exhibit metabolic effects in the brain. Our experiments confirmed that administration of pancuronium under constant isoflurane supply does not significantly change the brain lactate concentration (Supplementary Fig. 1).

No significant brain lactate increase under non-volatile anesthetics

To investigate whether reduced brain activity during isoflurane anesthesia elevates brain lactate, mice underwent 35 min without isoflurane (pancuronium only) before receiving either ketamine/medetomidine, medetomidine, pentobarbital, diazepam, or fentanyl. None of these narcotics led to a MRS-detectable level of brain lactate (Supplementary Fig. 7). Only pentobarbital caused a minor and transient lactate elevation.

Different HVA and pharmacokinetic–pharmacodynamic modeling

When using halothane, isoflurane, sevoflurane, or desflurane in different experimental protocols, the lactate concentration closely followed the respiratory concentrations of the respective HVA (Fig. 3). Lactate concentrations were best predicted by an effects compartment model accounting for the transfer of the anesthetic into the brain in conjunction with an indirect response model in which the lactate clearance was inhibited in a concentration-dependent saturable manner (see Materials and methods). According to this analysis the extrapolated basal brain lactate concentration was 0.24 mM with an elimination rate constant of 0.78 min^{-1} in brain tissue. The constant describing maximum inhibition of lactate was not significantly different between the four HVA. However, the respective concentrations resulting in 50% inhibition (IC50) of lactate clearance were 0.12, 0.13, 0.18, and 0.23% for isoflurane, halothane, sevoflurane, and desflurane. These four volatile anesthetic agents therefore differed in their potency to increase cerebral lactate. The IC50 values correlated (regression coefficient $r^2 = 0.87$) with the known minimal alveolar concentrations (MAC) of 1.2, 0.8, 2.0, and 6.0% for isoflurane, halothane, sevoflurane, and desflurane, respectively.

Isoflurane and adrenergic mechanisms

To further elucidate the underlying mechanisms, we applied different pharmacologic modulators during constant supply of isoflurane. The administration of medetomidine (0.5 mg/kg s.c.) led to a significant reduction of brain lactate by about 35% (Fig. 4). Medetomidine is an adrenergic $alpha_2$ receptor agonist which exhibits strong antiadrenergic effects in the central nervous system. Like lactate, the isoflurane-induced rises of alanine, choline-containing compounds, and *myo*-inositol were also abolished by medetomidine. Noteworthy, medetomidine effects were only detectable under isoflurane, whereas mice without isoflurane (pancuronium muscle relaxation only) and medetomidine did not differ from untreated animals (Supplementary Fig. 8).

The reduction of lactate by medetomidine was fully reversed by the administration of the $alpha_2$ receptor antagonist atipamezole (2.5 mg/kg s.c.). With atipamezole and constant isoflurane the lactate level even exceeded that observed under isoflurane alone (Fig. 4). Clonidine (5 mg/kg i.p.), another slightly less potent antiadrenergic presynaptic $alpha_2$ receptor agonist, also attenuated the isoflurane-induced lactate increase, although to a lower extent than medetomidine (Supplementary Fig. 9).

The role of adrenergic mechanisms as a contributor to isoflurane-induced increases of lactate is further supported by the fact that also the nonselective and CNS-active beta blocker propranolol significantly reduced the lactate increase (Fig. 5). Although quantitatively less pronounced, the noncompetitive NMDA receptor antagonist ketamine, known to inhibit several ionotropic glutamate receptors and to reduce the NMDA receptor dependent acetylcholine release, also caused a reduction of the lactate level under isoflurane (Fig. 5). This observation indicates that besides adrenergic mechanisms also glutamatergic mechanisms may be involved in the isoflurane-induced lactate increase.

Isoflurane and pyruvate dehydrogenase (PDH)

To address a possible alteration of PDH by isoflurane, mice received dichloroacetate (50 mg/kg i.p.), a common therapeutic in children with heritable mitochondriopathy activating the PDH. No significant reduction of the lactate level was observed during 100 minutes after dichloroacetate administration (Fig. 5).

Isoflurane and GABA

Administration of diazepam as a positive allosteric modulator of the $GABA_A$ receptor showed a tendency to attenuate the isoflurane-induced lactate increase ($p = 0.07$, Fig. 5). Interestingly, however, pentobarbital, likewise GABAergic but exhibiting multiple additional effects, resulted in a more than 3-fold increase of lactate under constant isoflurane (1.75%) and a lethal outcome unless isoflurane exposure was switched off (Fig. 6). In addition, alanine increased by a factor of two. After withdrawal of isoflurane but still under exposure to pentobarbital the lactate concentration dropped to the isoflurane-induced baseline level within about 45 min. Both diazepam and pentobarbital did not considerably alter cerebral GABA concentrations, whereas medetomidine, clonidine, and dichloroacetate, moderately reduced cerebral GABA under isoflurane (Fig. 5). Because of these observations, GABAergic effects are unable to fully explain the isoflurane effect on cerebral lactate.

Although medetomidine and pentobarbital showed almost opposite effects on lactate and alanine under isoflurane, both substances reduced the glutamate level (Figs. 4 and 6). These findings suggest that also glutamatergic mechanisms can not entirely explain the observed isoflurane effects.

Modulation of glucose, Cr and PCr under isoflurane

Although isoflurane-induced lactate could be reduced by several pharmacological manipulations, a significant increase of glucose was only seen for ketamine and diazepam. A reduction of creatine and a corresponding significant increase of phosphocreatine were only caused by propranolol (Fig. 5).

In summary, most of the isoflurane effects could partially be reversed by blockers of the adrenergic nervous system like medetomidine and propranolol. Stimulators of the adrenergic nervous system had the opposite effect by even further enhancing the isoflurane-induced lactate increase. These findings strongly indicate an activation of the adrenergic system by isoflurane.

Discussion

The key finding of this *in vivo* MRS study of mouse brain is that HVA such as isoflurane, halothane, sevoflurane, and desflurane at common anesthetic concentrations reversibly alter the concentrations of major brain metabolites. This particularly applies to a strong, rapid, and reversible increase of brain lactate which approaches levels as high as previously seen only under ischemic conditions (Schwarcz et al., 2003). The effect was most pronounced in the striatum and could significantly be attenuated by pharmacologic inhibitors of the central adrenergic nervous system. No significant correlation of the lactate increase was observed with age ($n = 60$, 29–548 d, $r^2 = 0.07$)

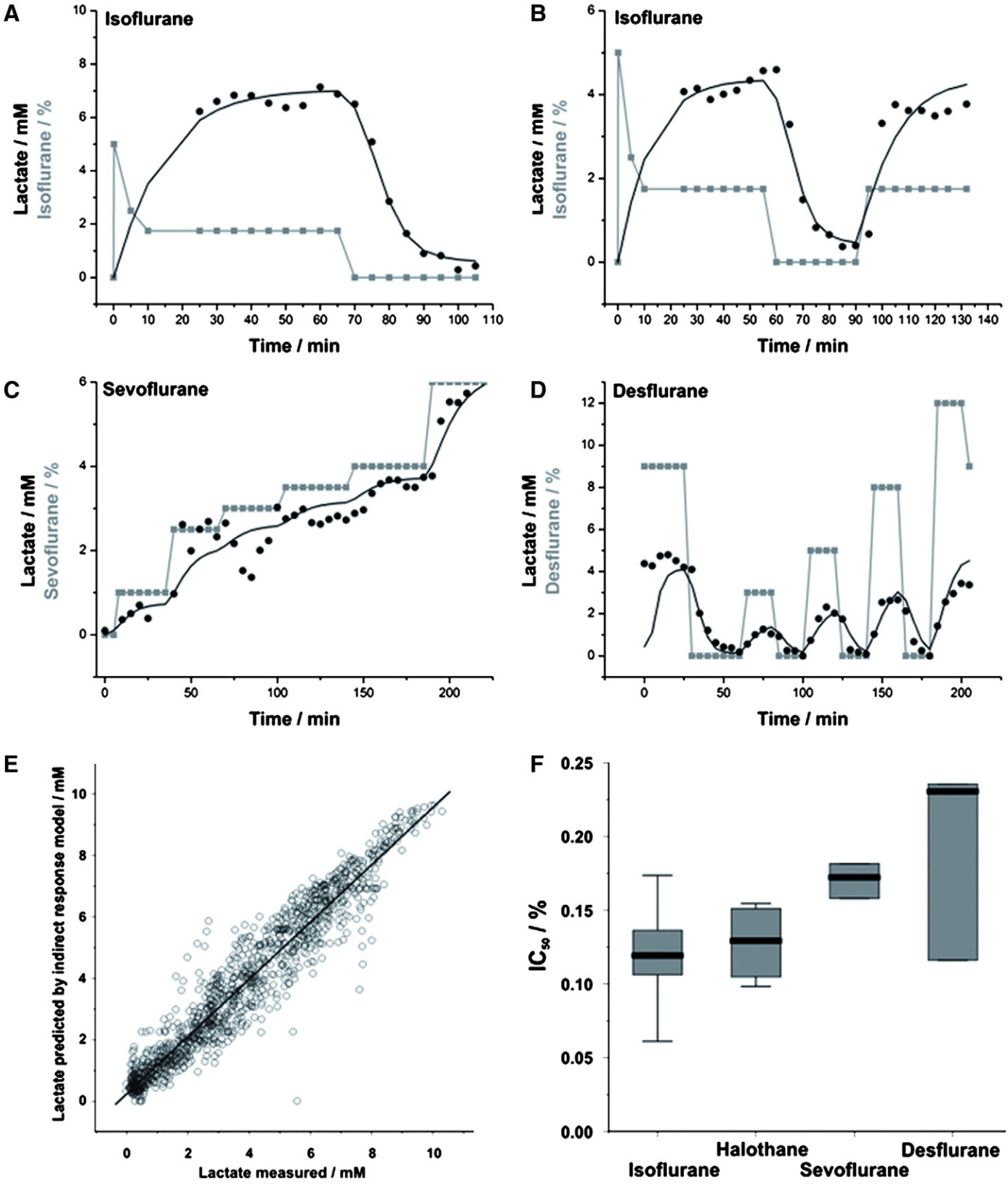

Fig. 3. Analysis of HVA-induced changes of cerebral lactate for different anesthetic protocols using pharmacokinetic–pharmacodynamic modeling. Applied isoflurane concentrations are shown by gray lines and symbols. (A–D) In all cases, the measured lactate concentration (black symbols) followed the HVA breathing air concentration and could be described by an indirect response model (solid lines, see Materials and methods). (E) The model shows residual scatter, but no systematic deviation between observed and predicted values. (F) Extrapolated intracerebral concentrations at 50% of maximum inhibition (IC50) of cerebral lactate clearance for isoflurane, halothane, sevoflurane, and desflurane.

or body weight (17.5–62.7 g, $r^2 = 0.07$). Similar results were found for male NMRI mice (data not shown) and in mice with a C57BL/6 background (Fünfschilling et al., 2012).

Possible mechanisms that may increase brain lactate include an increased uptake from the blood circuit, an increased catabolism of glucose to pyruvate, and a reduced metabolism of pyruvate via the

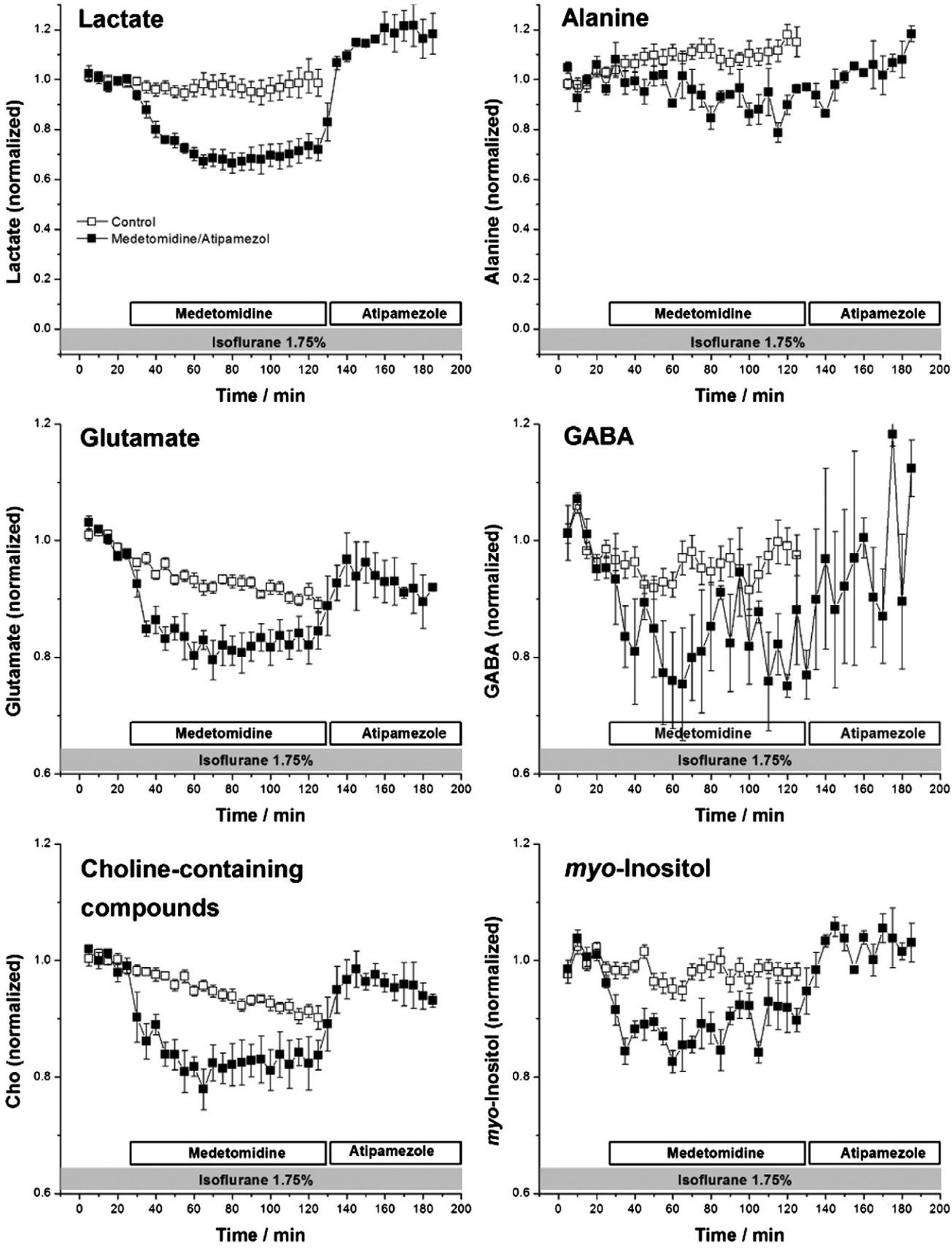

Fig. 4. Modulation of cerebral metabolites under isoflurane by medetomidine and atipamezole. Administration of medetomidine ($n=4$) caused a significant reduction of lactate, alanine, glutamate, choline-containing compounds (Cho) and *myo*-inositol (solid symbols, normalized to baseline, mean ± standard error). GABA was also reduced though with larger variability. The effects of the alpha$_2$ agonist medetomidine were reversed by the alpha$_2$ receptor antagonist atipamezole. Open symbols refer to cerebral metabolites under constant isoflurane without further medication ($n=11$).

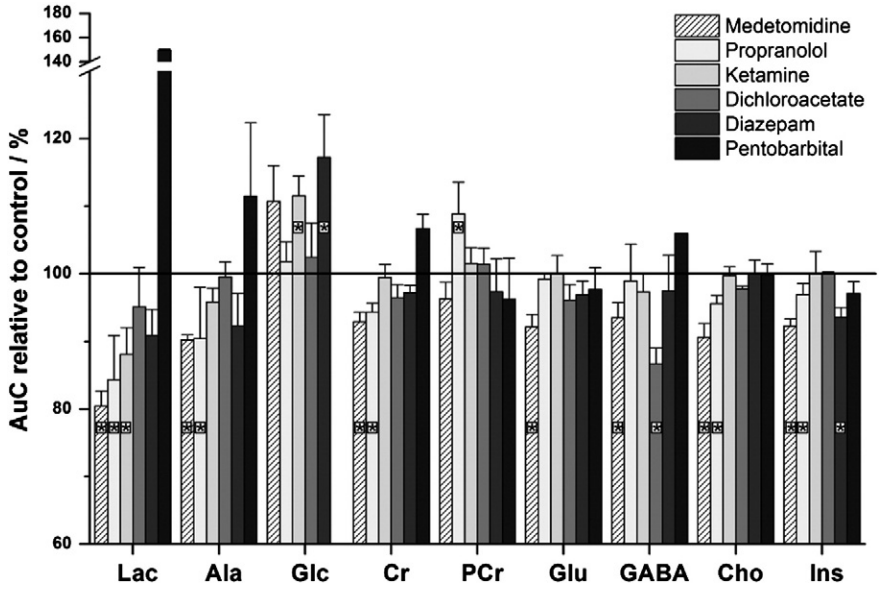

Fig. 5. Pharmacologic modulation of cerebral metabolites under isoflurane. Areas under the time curve (AuC) of metabolite concentrations (in percentage relative to values under 1.75% isoflurane, mean ± standard error) obtained before (25 min) and after additional administration of medetomidine ($n=4$), propranolol ($n=3$), ketamine ($n=4$), dichloroacetate ($n=3$), diazepam ($n=3$, all 75 min), or pentobarbital ($n=2$, 30 min). $^{*}p<0.05$ (*t*-test, controls vs. treated, excluding pentobarbital). Ala = alanine, Cr = creatine, Cho = choline-containing compounds, GABA = γ-aminobutyric acid, Glc = glucose, Glu = glutamate, Ins = *myo*-inositol, Lac = lactate, PCr = phosphocreatine. AuC of glucose were excluded for pentobarbital due to Cramer–Rao lower bounds above 20%.

tricarboxylic acid cycle (TCA). However, with reference to the first point, an increase of arterial lactate by anesthesia or lactate infusion did not significantly increase the lactate uptake into the brain (Kuhr et al., 1988; Quistorff et al., 2008; Rose, 2010). And while isoflurane leads to vasodilation and an increase of cerebral blood flow (Miller, 2000), metabolite signals from moving blood become dephased by gradient-localized MRS acquisitions and therefore do not contribute to *in vivo* MR spectra (Frahm et al., 1991).

Withdrawal of isoflurane led to an increase of brain glucose, which may be explained by an isoflurane-induced increase of glucose consumption via glycolysis or other pathways. In fact, ^{18}F-FDG positron emission tomography studies revealed a reduction of the cerebral metabolic rate of glucose under isoflurane (Alkire et al., 1997; Toyama et al., 2004). Because this technique measures both glucose uptake and hexokinase activity, the data suggest a reduced glucose uptake under isoflurane, although the uptake may still be higher than for other anesthetics as recently shown for thiopental (Duarte and Gruetter, 2012). In our study the infusion of glucose under isoflurane indeed increased brain glucose to a moderate degree, but the lack of a significant lactate increase argues against elevated glycolysis as the main cause of an isoflurane-induced lactate increase.

Although we tried to make the experimental setup as comfortable as possible, stress in anesthetic-free periods cannot completely be ruled out. Under stressful condition the uptake of glucose into the brain can be increased (Madsen et al., 1995) which may have contributed to the observed glucose increase after withdrawal of isoflurane. However, whereas a re-exposure to isoflurane again reduced the glucose concentration (Fig. 1), medetomidine anesthesia following an isoflurane-free period led to a further increase of glucose (Supplementary Fig. 10). This observation argues against stress as the main cause behind the increase in brain glucose after withdrawal of isoflurane.

As shown in cell culture, HVA can attenuate mitochondrial respiration and specifically affect the NADH dehydrogenase (Nahrwold and Cohen, 1973). Along the same line of evidence, HVA increased the accumulation of reactive oxygen species *in vitro* and *in vivo* (Zhang et al., 2011) and reduced the cerebral metabolic rate of oxygen (Michenfelder and Theye, 1975). An alteration of the mitochondrial pathway by HVA is further supported by our observation of the highest lactate levels in the striatum. Compared to the cerebral cortex which exhibits the lowest lactate level under isoflurane, the striatum is known to be particularly vulnerable to mitochondrial toxins which is supported by an increase of striatal lactate after exposure to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridin (Koga et al., 2006).

Another indication for an impairment of the respiratory chain by isoflurane is the observed shift of phosphocreatine towards creatine, which is in line with diminished brain energy stores (ATP, PCr) by halothane observed in *post mortem* studies of dogs (Michenfelder and Theye, 1975). Moreover, dichloroacetate, which is known to enhance the activity of the PDH complex (Stacpoole et al., 1998) did not significantly reduce brain lactate pointing to alterations in later stages of the mitochondrial pathway.

A recent study revealed a cAMP signaling cascade in mitochondria (Acin-Perez et al., 2011) with cAMP as activator of the respiratory chain. Moreover, it has been shown that formation of cAMP is stimulated by CO_2. We indeed observed a strong reduction of lactate under isoflurane by increasing the CO_2 level of the breathing air by up to 30% (Supplementary Fig. 11). However, in view of the known vasodilating effects of CO_2 and its putative enhancement of lactate clearance from brain tissue, the HVA modulation of mitochondrial cAMP-mediated pathways has to be clarified in further studies.

HVA are known to affect the GABAergic and glutamatergic pathways (Arai et al., 1990; Bickler et al., 1994; Larsen et al., 1994; Rau et al., 2011; Rudolph and Mohler, 2004; Zhao et al., 2011), for example by inhibiting glutamate release and enhancing glutamate uptake and GABA release (Westphalen and Hemmings, 2006). We observed an increase of GABA and glutamate under isoflurane as well as a slight but constant decrease of glutamate with time (Fig. 1), which was particularly evident for a 2 hour exposure to isoflurane (Supplementary Fig. 1). No changes in glutamine were observed (Table 2, Supplementary Fig. 3). Both, diazepam and pentobarbital are enhancers of GABAergic neurotransmission, but the first led to a reduction and the latter to an extensive increase of lactate (Figs. 5 and 6). Similarly, the reduction of glutamate by medetomidine and pentobarbital led to contradictory lactate concentrations. Together, these results suggest that the isoflurane-induced increased brain lactate is not mediated

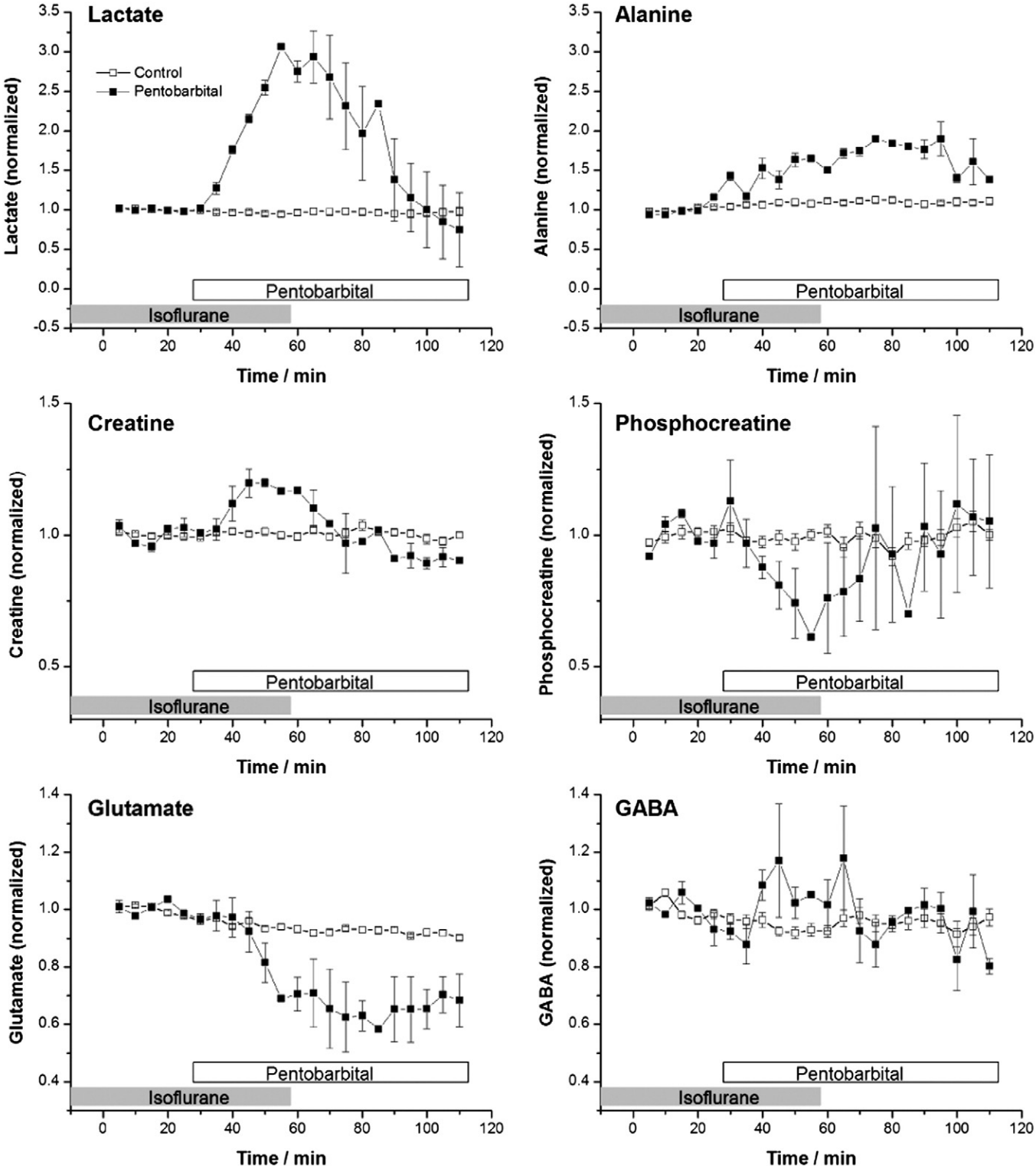

Fig. 6. Modulation of cerebral metabolites under isoflurane by pentobarbital. Administration of pentobarbital resulted in a 3-fold increase of lactate, which was reversible after isoflurane withdrawal. In addition, alanine increased twofold, creatine increased by 20% and GABA increased by about 10% although with larger variability, whereas glutamate and phosphocreatine decreased (mean ± standard error, $n = 2$).

via the neurotransmitters GABA or glutamate. More likely, isoflurane seems to alter the oxidative metabolism of these neurotransmitters by disturbing mitochondrial functions. Fig. 7 schematically outlines the metabolic pathways involved and the isoflurane-induced changes observed here.

An unexpected observation was the fact that choline-containing compounds and *myo*-inositol decreased within minutes after withdrawal of isoflurane and increased again after isoflurane exposure. Isoflurane is known to affect the cholinergic system including its binding to acetylcholine receptors (Xu et al., 2000). It also decreases

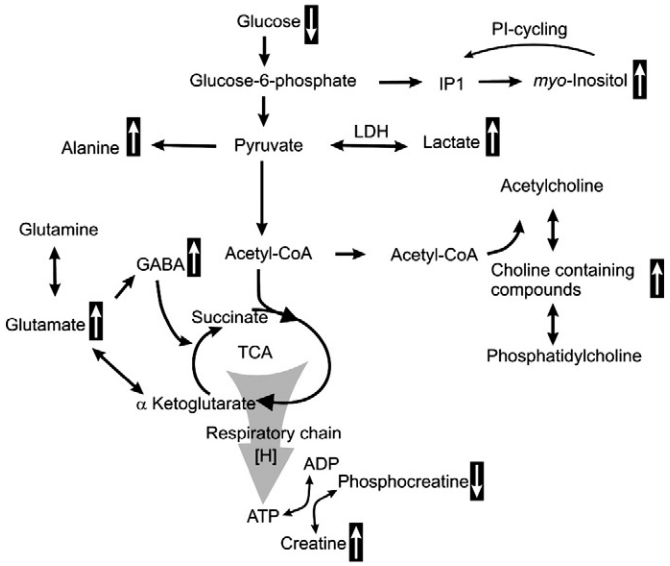

Fig. 7. Schematic diagram of isoflurane effects on cerebral metabolism. Isoflurane causes increases of lactate, alanine, creatine, GABA, glutamate, choline-containing compounds, and *myo*-inositol as well as a decrease of glucose and phosphocreatine (arrows). A possible mechanism for brain lactate increases refers to a reduced metabolism of pyruvate via the tricarboxylic acid cycle (TCA). ADP: adenosine diphosphate, ATP: adenosine triphosphate, CoA = coenzyme A, IP1 = inositol-monophosphate, PI = phosphatidylinositol, LDH = lactate dehydrogenase.

the release of acetylcholine (Shichino et al., 1997) and the mean opening of the acetylcholine receptor channel (Miller, 2000). However, the brain acetylcholine concentration (Buxton et al., 1976) is at least one order of magnitude lower than the observed changes in choline-containing compounds. Therefore, degradation of phosphatidylcholine or other reversible changes of cell membranes are a more likely explanation for the respective increase under isoflurane. This is in line with the observation that isoflurane increases the diffusivity of several metabolites including choline-containing compounds (Valette et al., 2007). For rat hepatocytes it has been shown that norepinephrine (via α-adrenergic receptors) decreases the incorporation of choline into phoshatidylcholine (Haagsman et al., 1984). Remarkably, administration of medetomidine, which inhibits the release of norepinephrine, reversed the elevation of choline-containing compounds by isoflurane suggesting a comparable adrenergic regulation of phoshatidylcholine synthesis in the brain.

Cerebral *myo*-inositol is synthesized *de novo* from glucose-6-phosphate and is a precursor in the PI cycle second messenger system (Kim et al., 2005). As shown by studies in cell cultures isoflurane activates the inositol-1,4,5-triphosphate (IP_3) receptor (Wei et al., 2008). Because the PI cycle can be activated via $alpha_1$ and $alpha_2$ adrenergic receptors, for instance by norepinephrine, this may explain the reduction of *myo*-inositol under isoflurane by medetomidine.

As discussed above, most of the metabolic effects observed under isoflurane were attenuated by the presynaptic $alpha_2$ adrenergic receptor agonist medetomidine (Fig. 4). Likewise, the metabolic effect of medetomidine was reversed by the $alpha_2$ receptor antagonist atipamezole, which even partly enhanced the isoflurane effects. Interestingly, mice lacking the $alpha_{2A}$ adrenoreceptor showed a reduced antinociceptive response to isoflurane (Kingery et al., 2002). These findings strongly suggest an action of isoflurane on the adrenergic system with a direct or indirect involvement of the $alpha_2$ adrenergic receptor. However, medetomidine could not completely reverse the isoflurane-induced lactate increase to the low isoflurane-free level and metabolic changes were also, though partially, compensated by other pharmacologic modulators (Figs. 4 to 6). Thus, further mechanisms such as effects on oxidative energy metabolism are likely to contribute to the isoflurane-induced alterations. Noteworthy, the potency of the different HVA in increasing cerebral lactate and their correlation with known MAC values suggest a mechanistic link between HVA-induced lactate and anesthetic effects.

A surprising observation was the rapid reversibility of the metabolic effects of all HVA tested. Major changes of lactate and alanine as well as smaller effects such as seen for glucose closely followed the applied concentration of isoflurane (Figs. 1 and 3). In particular, the rapid changes in lactate may support the recently proposed role of lactate as a mediator of metabolic information (Bergersen and Gjedde, 2012). Moreover, considering the known neuroprotective properties of isoflurane, these findings with quite high lactate under isoflurane may cast new light on the role of lactate in stroke or neurotrauma, supporting the role of lactate as an energy substrate in the injured brain rather than considering lactate as a tissue-damaging or damage-enhancing substance.

Apart from the acute isoflurane effects, there were also longer-lasting alterations such as the incomplete recovery of lactate and alanine levels after a second exposure to the same concentration of isoflurane (Fig. 1). This also applies to the steady decline of glutamate over a period of more than 2 hours (Supplementary Fig. 1). These observations may be due to an accumulation of a small HVA fraction in so-called deep compartments or other yet unknown modulations of signaling pathways.

The strong correlation between HVA concentration and multiple metabolic changes may affect the interpretation of functional and metabolic brain studies under HVA anesthesia. This may be even more critical when delivering isoflurane via a mask with spontaneous breathing, which may lead to larger variations of the isoflurane concentration between animals.

In conclusion, isoflurane and other HVA cause rapid, pronounced, and mostly reversible changes in cerebral metabolites as assessed in mouse brain *in vivo*. These effects occur at concentrations commonly applied in human medicine and in animal research and therefore may have multiple implications. Most importantly, HVA influences should be known and considered in any functional brain imaging study under HVA. In addition, the present data from *in vivo* MRS cast new light on possible mechanisms of action — of HVA alone or in combination with other drugs.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.neuroimage.2012.12.020.

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