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1 Prediction of Neuroprotective Treatment Efficiency Using a HRMAS 2 NMR-Based Statistical Model of Refractory Status Epilepticus on 3 Mouse: A Metabolomic Approach Supported by Histology

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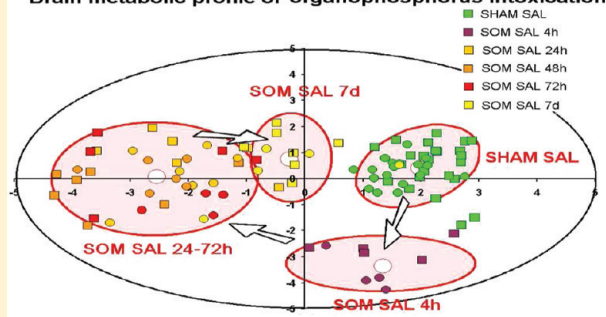
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8 **ABSTRACT:** This work presents a model combining quantitative proton HRMAS NMR data and PLS-DA for neuropathology and neuroprotection evaluation. Metabolic data were also confronted to histopathological results obtained using the same experimental conditions. Soman, when not lethal, can induce status epilepticus (SE), brain damage, histological lesions, and profound cerebral metabolic disorders as revealed using ¹H HRMAS NMR. Our challenge was to evaluate delayed treatments, which could control refractory SE and avoid brain lesions. For this aim, we have built a statistical model of soman intoxication describing brain metabolite evolution during 7 days.

19 We have then used this model to evaluate the efficiency of a combination of ketamine/atropine (KET/AS) administrated 1 and 2 h after SE induction, compared to the immediate anticonvulsant therapy midazolam/atropine sulfate (MDZ/AS). Furthermore, quantitation of HRMAS NMR data allowed us to follow individual evolution of 17 metabolites. *N*-Acetylaspartate, lactate, or taurine presented a long lasting disruption, while glutamine, alanine, glycerophosphocholine and myo-inositol showed disruptions for 3 days with a reversion at day 7. These changes were completely normalized by the administration of MDZ/AS. Interestingly, they were also almost completely reversed by KET/AS 1 h postsoman. This work suggests further the predictive interest of HRMAS and PLS-DA for neuropathology/neuroprotection studies and also confirms, on the metabolic aspects, the neuroprotective potentials of KET/AS combinations for the delayed treatment of soman-induced SE.

28 **KEYWORDS:** soman, ketamine, midazolam, high resolution magic angle spinning, PLS-DA, brain histology

Brain metabolic profile of organophosphorus intoxication



29 ■ INTRODUCTION

30 Soman is an organophosphorus nerve agent that acts as an irreversible inhibitor of acetylcholinesterase both in central and in peripheral nervous systems. This leads to a massive hypercholinergy resulting in hypersecretion, respiratory distress, convulsive seizures and rapid death if the dose is sufficient.¹ Although soman-induced seizures primarily stem from cholinergic overstimulation,² their maintenance is mainly attributable to a secondary massive release of glutamate,¹ an excitatory amino acid that has been recognized for its "excitotoxic" potential.³ In survivors, seizure activity may evolve into a *status epilepticus* (SE) that becomes refractory to benzodiazepines if not quickly stopped. Numerous sensitive brain areas such as cortex, piriform cortex, amygdala, septum, thalamus or hippocampus may end up showing seizure-related brain damage (SRBD).^{4–7} Survivors of these severe intoxications may suffer from neurological sequelae such as spontaneous recurrent seizures⁸ after a latent period of epileptogenesis, as well as cognitive deficits.^{9,10} Numerous pathological events are known to accompany SRBD. For instance, a cytotoxic and vasogenic cerebral edema develops

within a few hours after poisoning^{11,12} and neuroinflammation occurs in the hours or days following SE as evidenced through the appearance of astro- and microgliosis,^{4,13} recruitment of peripheral immune cells^{14,15} and synthesis of various molecular markers such as cytokines and cell adhesion molecules.^{16–18}

Moreover, SE, would it be soman-induced or of a different origin,^{19–21} can trigger major disturbances of brain metabolism including changes in the energy,^{22,23} aminoacid²⁴ and phospholipid metabolisms.^{25,26} To explore further cerebral metabolic changes after soman, we recently used proton high resolution magic angle spinning (¹H HRMAS) nuclear magnetic resonance (NMR) spectroscopy (HRMAS NMR).²⁷ This technique, which has been successfully applied in various tissues and diseases,^{28–31} allows the characterization of the metabolic phenotypes of cells, tissues and organs under both normal and pathological conditions, without any extraction or chemical treatments. Therefore, it offers the advantage of eliminating the contamination associated with extraction

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68 procedures. With this method applied *ex vivo* to brain biopsies
69 from mice, we were able in our previous work to quantitate
70 using the jMRUI software (www.jmrui.uab.es/mrui/) 17
71 metabolites up to 7 days after soman-induced SE.²⁷ While
72 only 2 metabolites were modified in the cerebellum, a structure
73 that apparently remains structurally intact in soman-intoxicated
74 mice, 13 showed significant changes in the piriform cortex. In
75 the latter, changes could be detected as soon as 4 h
76 postchallenge, but with the notable exception of *N*-
77 acetylaspartate (NAA), lactate and taurine, metabolite levels
78 were normalized after one week. Some of the observed
79 metabolic disorders could be tentatively related to postsoman,
80 SE-related pathophysiological events.²⁷ For instance, NAA
81 decrease may be considered as a marker of neuronal loss or
82 neuronal mitochondrial dysfunctions.³² The large increase of
83 lactate observed from 24 to 72 h after intoxication could also be
84 associated with mitochondrial dysfunction.³³ Myo-inositol and
85 taurine are among the main osmolytes in the brain, and their
86 decrease could be related to the observed brain edema.^{34,35}
87 Glycerophosphocholine increase could be associated to
88 phospholipase activation during neuroinflammatory process
89 induced by seizures.³⁶

90 In this previous work,²⁷ HRMAS NMR quantitative data
91 were also submitted to multivariate statistical analysis. These
92 methods, such as principal component analysis (PCA) and
93 projection to latent structure-discriminant analysis (PLS-DA),
94 allow one to reduce the number of variables (i.e., 17 NMR
95 variables in our case) to two or three supervariables (latent
96 variables) that define a new space in which observations are
97 projected. This provides an important help to describe data and
98 to find “metabolic profiles” characteristic of a given status or
99 pathology. Moreover, if the model is sufficiently robust, it could
100 allow predicting the classification of an unknown observation
101 from its NMR spectrum. We clearly showed metabolic
102 differences between damaged and less damaged brain structures
103 and helped to globally assess the time course of metabolic
104 changes. This study, bridging soman-induced SRBD and
105 metabolic disorders, suggested that quantitation of HRMAS
106 NMR data associated with PLS-DA analysis might be useful to
107 provide temporal and local metabolic signatures of soman
108 poisoning, and possibly markers for neuropathology and clues
109 to explain pathophysiological mechanisms.

110 The standard treatment against organophosphorus intox-
111 ication combines an antimuscarinic drug such as atropine
112 sulfate (AS) and an oxime that reactivates the inhibited
113 cholinesterase (e.g., HI6). Benzodiazepines like diazepam or
114 midazolam (MDZ) are used to stop seizures. The combination
115 of HI6, AS and MDZ (MDZ/AS) constitute the “antidote”
116 used by the French army, but this combination needs to be very
117 quickly administered, preferably within ca. 20 min after seizure
118 onset, to provide the best protection against SRBD and long-
119 term neurological deficits.^{37–41} Indeed, if the continuous
120 seizures are left unabated, they will not respond well to
121 benzodiazepines, and the SE becomes refractory. The manage-
122 ment of refractory SE (RSE) is a therapeutical challenge and
123 constitutes one of the main goals of our research.

124 Drug combinations of ionotropic glutamate receptor
125 antagonists (NMDA antagonists or AMPA antagonists) are
126 among the most efficient drugs. In experimental soman
127 poisoning and SE, NMDA antagonists such as MK-801,^{42,43}
128 TCP,^{44,45} GK-11⁴⁶ showed promising effects, most of the time
129 when associated with AS. Unfortunately, these drugs are either
130 not usable in humans because of severe adverse effects or have

not been licensed. Ketamine (KET) is the only NMDA 131
antagonist licensed as an injectable drug in different countries. 132
It is commonly used in emergency care and military 133
anesthesiology. It has little effect on the cardiovascular system 134
and does not easily induce respiratory depression. It is 135
recognized as a possible third line treatment of RSE.^{47,48} It 136
also exerts peripheral anti-inflammatory properties.^{49,50} In 137
guinea pigs, KET in combination with AS was shown to stop 138
seizures and drastically reduce brain damage even when 139
administered ca. 1 h after seizure onset,⁵¹ a property shared 140
by the active isomer S(+) KET.⁵² With a comparable paradigm, 141
KET/AS combinations were also shown to reduce brain 142
damage and neuroinflammation in poisoned mice.¹⁵ However, 143
KET/AS administered 2 h after soman provided very limited 144
neuroprotective effects.⁵¹ 145

These data show that KET combinations could constitute an 146
efficient delayed treatment of soman intoxication when SE 147
becomes refractory to benzodiazepine treatment. In this study 148
we have used a metabolomic approach to evaluate the effect of 149
KET treatments on brain damage. For this aim, we first built a 150
robust multivariate statistical “soman model”. Statistical power 151
was increased by combining new metabolic data with data of 152
our previous study.²⁷ Second, three treatments were tested: the 153
classical “antidote” administered immediately after soman 154
(MDZ/AS) and two delayed treatments with KET/AS at 1 155
and 2 h. The MDZ/AS treatment constituted a positive control 156
to evaluate the predictability of our statistical “soman model”, 157
which was then used to evaluate efficiency of delayed KET 158
treatments. In parallel to the HRMAS NMR analysis, a classical 159
histological study was performed. 160

Furthermore, the quantitation of HRMAS NMR data 161
allowed us to associate metabolic disorders to the various 162
events occurring during and after the intoxication, and to 163
identify more precisely the mechanism of action of treatments. 164

Finally, this study could contribute to support the use of 165
HRMAS NMR-based metabolomics as a predictive tool for 166
neuropathology and neuroprotection in preclinical studies. 167

168 ■ MATERIALS AND METHODS

169 Animals

Male Swiss mice (32.9 ± 2.0 g; Elevage Janvier, France) were 170
used in the present study. They were housed in a controlled 171
environment (21 ± 2 °C; 12 h dark/light cycle with light 172
provided between 7 a.m. and 7 p.m.). Food and water were 173
given *ad libitum*. All procedures followed were in accordance 174
with the regulations regarding the “protection of animals used 175
for experimental and other scientific purposes” from the 176
relevant Directive of the European Community (86/609/CEE) 177
and French legislation. The present protocol was approved by 178
the Ethical Committee of our Institute. 179

180 Drugs

Soman (>97% pure as assessed by gas chromatography) was 181
supplied by the Centre DGA maîtrise NRBC du Bouchet (Vert- 182
le-Petit, France). Solutions were freshly prepared by diluting 183
the initial stock solution (2 mg/mL in isopropanol) in 0.9% 184
(w/v) saline prior to the injection. The oxime HI6 dichloride 185
was a generous gift of DRDC Suffield (Canada). Methyla- 186
tropine nitrate and atropine sulfate (AS) were purchased from 187
Sigma Chemicals (France). Midazolam (MDZ) and ketamine 188
(KET) were purchased from Panpharma SA (France). 189

190 Experimental Design

191 Mice were pretreated with HI6 dichloride (50 mg/kg i.p.) and
192 methylatropine nitrate (4 mg/kg i.p.), 5 min prior to either
193 soman (172 $\mu\text{g}/\text{kg}$ ca. 0.6 LD_{50} in the presence of HI-6
194 pretreatment, s.c. in the nape of the neck) or saline injection
195 (200 μL , s.c.). The oxime HI6 dichloride, an acetylcholinester-
196 ase reactivator, and methylatropine nitrate, an antagonist of
197 peripheral muscarinic receptors, were used to limit lethality due
198 to respiratory distress.

199 This protocol is identical or very similar to those routinely
200 used in our laboratory^{4,11,15,16,27} and can serve as a model for
201 RSE with a high survival rate in absence of any therapy. For the
202 two challenge conditions, either intoxication with soman (SOM
203 groups) or no intoxication (SHAM groups), four different
204 treatment protocols were considered, leading to eight groups:

- 205 (1) Classical “antidote” treatment (MDZ/AS): SOM MDZ/
206 AS and SHAM MDZ/AS groups. This protocol
207 efficiently controls seizures when administered immedi-
208 ately after challenge. MDZ (25 mg/kg) and AS (10 mg/
209 kg) were administered i.p. only once in two separate
210 injections.
- 211 (2) Ketamine 1 h treatment (KET1h/AS): SOM KET1h/AS
212 and SHAM KET1h/AS groups. This protocol controls
213 the RSE when administered for the first time 1 h after
214 challenge. KET (100 mg/kg) and AS (10 mg/kg) were
215 administered in two separate injections 1 h after
216 intoxication. At 2 and 3 h postchallenge, KET (50 mg/
217 kg) and AS (10 mg/kg) were injected i.p. in two separate
218 injections.
- 219 (3) Ketamine 2 h treatment (KET2h/AS): SOM KET2h/AS
220 and SHAM KET2h/AS groups. This protocol does not
221 properly control the refractory SE when administered 2 h
222 after challenge. The same protocol as KET1h was used
223 but started 2 h after challenge.
- 224 (4) Control protocol: SOM SAL and SHAM SAL groups.
225 Three injections of saline (200 μL i.p.) replaced the
226 treatments, one injection every hour starting 1 h after
227 challenge (to match the KET/AS groups where the
228 treatments were administered three times).

229 All animals were observed for 6 h postchallenge, and the
230 signs and symptoms of the intoxication were recorded. Mice
231 were weighed just before any treatment and every day
232 postchallenge until euthanized. The body weight loss after
233 soman poisoning may be considered as a good indicator of the
234 global health status of the mice and a surrogate marker of
235 central damage.^{9,15,52,53}

236 Histological Analysis

237 For this study, no SHAM groups were considered. Four groups
238 ($n = 21$ each) were intoxicated with soman and received one of
239 the treatments previously described (SOM SAL, SOM MDZ/
240 AS, SOM KET1h/AS, SOM KET2h/AS). For each group, the
241 animals were euthanized either 24 h ($n = 7$), 72 h ($n = 7$) or 7
242 days ($n = 7$) after intoxication. Mice were deeply anesthetized
243 (sodium pentobarbital, 80 mg/kg; i.p.) and transcardially
244 perfusion-fixed with a mixture of formaldehyde (4%) and
245 acetic acid (3%). Coronal blocks of brain, from +2 to -2.5 mm
246 (containing the piriform cortex and the amygdala), and from
247 -5.5 to -7 mm (containing a part of the cerebellum) relative
248 to bregma, were embedded in paraffin. Histological sections (7
249 μm thick) chosen for analysis were situated approximately
250 either -1.94 or -6 mm relative to bregma.⁵⁴ They were stained

with Hemalun–Phloxin (HP) for the detection of damaged 251
(eosinophilic) cells, appearing in dark violet. On adjacent 252
sections, reactive astrocytes were revealed by glial fibrillary 253
acidic protein (GFAP) immunohistochemistry using a protocol 254
previously described.¹⁵ 255

Equally magnified (20 \times), high resolution (1.25 Mpixels, 48 256
bit colors), digital images (690 \times 520 μm) from the amygdaloid 257
nucleus, the piriform cortex and the dorsal cerebellar lobule 258
from each mouse were acquired with an Axio Imager Z1 Zeiss 259
microscope equipped with an Axiocam MR5 camera (Zeiss, 260
Germany). Eosinophilic cells and astrocytes were identified on 261
the basis of their color, shape and size and quantitated using the 262
Axiovision image processing software (vs 4.6.3.0). Quantitation 263
was achieved as previously described:¹⁵ within each chosen 264
brain structure, the number of eosinophilic cells on the HP- 265
stained sections and the percentage of the surface occupied by 266
GFAP positive cells on the GFAP-treated sections were 267
determined. The results are expressed as the mean \pm SEM 268
calculated for each group and at each time point. 269

¹H HRMAS NMR Spectroscopy 270

Biopsy Preparation. For each group and each time point, 271
5–9 mice depending on the group size were euthanized 4, 24, 272
48, or 72 h and 7 days after challenge. Mice were decapitated, 273
and their brains were rapidly removed and dissected on ice for 274
the preparation of HRMAS biopsies. Two samples were 275
chosen: the first contained the piriform cortex and amygdala 276
(PIR/AMG) from both hemispheres, as severely damaged 277
areas; the second was the cerebellum (CER), an area that 278
remains apparently structurally intact. These biopsies were 279
obtained using a coronal dissection matrix allowing the 280
dissection of slices (2–3 mm thick) located from +0.5 to 281
-2.5 mm (for PIR/AMG) or from -5.5 to -7.5 mm (for 282
CER) relative to the bregma. They were immediately frozen in 283
liquid nitrogen. Approximately 15 mg of the frozen biopsies 284
were used for each assay. 285

HRMAS Data Acquisition. Proton NMR spectra were 286
recorded on a Bruker Avance 400 spectrometer (proton 287
frequency 400.13 MHz) equipped with a 4 mm ¹H–¹³C–³¹P 288
HRMAS probe-head. Samples were spun at 4000 Hz. 1D 289
spectra were all acquired with a Carr–Purcell–Meiboom–Gill 290
(CPMG) pulse sequence to attenuate macromolecule and lipid 291
resonances, synchronized with the spinning rate (interpulse 292
delay 250 μs , total spin echo time 30 ms). Residual water signal 293
was presaturated during the 2 s relaxation delay time. Total 294
acquisition of one spectrum with 256 scans lasted 16 min. 295
Resonance assignment was performed as previously de- 296
scribed.⁵⁵ 297

Data Processing. Quantitation was performed with the 298
package software jMRUI using the “subtract-QUEST” 299
procedure.⁵⁶ This procedure uses a simulated metabolite 300
database (for details, see Rabeson⁵⁵). Twenty-one metabolites 301
were included in the database: acetate (Ace), alanine (Ala), 302
aspartate (Asp), creatine and phosphocreatine (Cr), choline 303
(Cho), ethanolamine (Eth), γ -amino-butyric acid (GABA), 304
glutamate (Glu), glutamine (Gln), glutathione (Gsh), glycer- 305
ophosphocholine (GPC), glycine (Gly), lactate (Lac), myo- 306
inositol (M-ins), *N*-acetylaspartate (NAA), phosphoethanol- 307
amine (PE), phosphocholine (PC), scyllo-inositol (S-ins), 308
serine (Ser), taurine (Tau) and valine (Val). The amplitude 309
of each metabolite signal calculated by QUEST was normalized 310
to the total spectrum signal. The Cramer Rao lower bounds 311
(CRLB) calculated by the jMRUI algorithm are estimates of the 312

313 standard deviation of the fit for each metabolite. For 17
314 metabolites, CRLB was either ≤ 5 or $\leq 25\%$ (Ace, Asp). They
315 were all included in the statistical analysis. Only four
316 metabolites (Eth, S-ins, Ser and Val), showed a CRLB \geq
317 50% and were excluded from the statistical analysis.

318 Statistical Analysis

319 **Univariate Statistical Analysis.** All metabolic data, image
320 quantitations or body weight changes are expressed as mean \pm
321 SEM. Data were analyzed using the Kruskal Wallis ANOVA by
322 ranks to compare the different post treatment groups. These
323 tests were followed by a post hoc multiple comparison test: at
324 each time, treated groups (MDZ/AS, KET1h/AS, KET2h/AS)
325 were compared to the SHAM groups or to the intoxicated,
326 untreated mice (SOM SAL group) (Statistica v. 6.1 software,
327 Statsoft, Inc.). The level of significance was set at $\alpha = 5\%$.

328 **Multivariate Statistical Analysis. Statistical Model of**
329 *Soman Intoxication ("Soman Model").* The statistical model
330 of soman intoxication, named "soman model", was built with
331 data from the SOM SAL and SHAM SAL groups and from our
332 previous study.²⁷ The only difference between the two studies
333 consists in the injection of saline in the present work, which was
334 not used in the previous one. The amplitude of each metabolite
335 quantitated after HRMAS analysis were then imported into the
336 SIMCAP V12 software (Umetrics AB, Umea, Sweden) as X
337 variables. They were mean centered, scaled to unit variance
338 (i.e., weighted by $1/\text{standard deviation}$ for a given variable) and
339 grouped in a first principal component analysis (PCA),
340 resulting in a total matrix of 102 observations (mice) and 17
341 variables. This allows us to rapidly evaluate quality and
342 homogeneity of data and to find clusters for further supervised
343 statistical analysis. It appeared that the observations of the
344 previous²⁷ and present HRMAS studies were well grouped,
345 even if some differences could be observed for some variables
346 individually (see the Results section). Consequently, all data
347 were included in the model to increase its statistical power.

348 Then partial least square discriminant analyses (PLS-DA)⁵⁷
349 were run with the clusters that appeared in PCA analysis as Y
350 variables, in order to build a model that optimizes the
351 separation between them. The number of components was
352 determined using the cross validation procedure that produces
353 R^2Y and Q^2 factors. The R^2Y value indicates the goodness of fit,
354 while the Q^2 value is a measure of the predictability of the
355 model. It is generally considered that $R^2Y > 0.5$ and $Q^2 > 0.5$
356 are decent values.

357 In order to obtain an estimate of the significance of the Q^2
358 value, the model was validated using random permutations of
359 the Y matrix (999 permutations). For each permuted Y , Q^2 is
360 recalculated, and the correlation coefficients between the
361 original Y and the permuted Y versus the cumulative Q^2 are
362 represented by a regression line whose value to the origin must
363 be negative, indicating the absence of any overfitting in the
364 model.⁵⁸ The same procedure is applied to R^2Y values.

365 Moreover, the reliability of our PLS-DA model was assessed
366 by a CV-ANOVA test (analysis of variance testing of cross-
367 validated predictive residuals). With this method, the
368 metabolites also received a variable importance in the
369 projection (VIP) that allows ranking them according to their
370 contribution to the model.

371 *Prediction of Treatment Efficacy Using the "Soman*
372 *Model".* Finally, we compared the output of the model with
373 the actual results obtained following the administration of the
374 neuroprotective treatments. Treated animals, intoxicated or

not, were imported in the model with no a priori class 375
assignment, and the score scatter plots drawn for graphical 376
inspection. These observations were classified using the vicinity 377
criterion. Moreover, the classification rate (in percentage) in 378
the SHAM SAL group was produced to evaluate the efficacy of 379
the treatment. 380

381 RESULTS

382 Clinical Course of the Animals

383 Mice from SHAM SAL group did not present any behavioral 383
modification after any of the injections. Mice from the SHAM 384
MDZ/AS group presented a sedation lasting 3–4 h after 385
treatment, and mice from KET/AS groups presented sedation 386
between the first and the second injection and during 30–45 387
min after the second and the third injection. The first KET 388
injection was performed with a high anesthetic dose (100 mg/ 389
kg). Despite the use of a reduced dosage for the second and 390
third administration, 8% of the mice treated with the KET/AS 391
protocol alone died, presumably from a KET overdose or 392
because of the combination with AS. 393

394 Whatever their treatment, SHAM mice only showed a very 394
limited loss of their body weight at 24 h followed by complete 395
recovery of the prechallenge weight at 48 h. Weight then 396
regularly increased during the following days. 397

398 The immediate administration of an anticonvulsant therapy 398
(MDZ/AS) to soman poisoned mice prevented the appearance 399
of convulsions and induced a quick sedation. Only small 400
fasciculation could be observed in 60% of the mice. Their body 401
weight loss was limited (ca. 10% at 24 h), and a complete 402
recovery of body weight was observed at day 7. 403

404 Conversely, without the MDZ/AS treatment (animals from 404
the groups SOM SAL, SOM KET1h/AS and SOM KET2h/ 405
AS), strong continuous convulsions appeared 3–8 min after 406
soman intoxication in 80% of the mice, while 20% presented 407
discontinuous convulsions. When no treatment was given 408
(SOM SAL group) convulsions lasted for 4–8 h with a 409
decreasing intensity. Twenty-five percent of these animals died 410
between 10 min to 6 h after intoxication. The survivors 411
experienced a drastic body weight loss (Figure 1), reaching 412
about -20% two days postchallenge and very poor recovery in 413
the following days (-12% at day 7). All mice from the SOM 414
KET1h/AS and SOM KET2h/AS groups convulsed until the 415
first injection of KET; convulsions then disappeared when 416
sedation started. In some mice of the SOM KET2h/AS group, 417
convulsions reappeared when the sedation waned between two 418
subsequent KET injections. 7 and 9% of the mice from SOM 419
KET1h/AS and SOM KET2h/AS groups died from intox- 420
ication before the first injection of KET/AS, and 7 and 14% 421
died after KET/AS treatment. Interestingly, the body weight 422
loss of surviving animal treated 1 h after soman (SOM KET1h/ 423
AS) was similar to that of the MDZ/AS group (ca. -10% at 24 424
h) but with only a partial recovery (-4% at day 7). Conversely, 425
the body weight loss of animal treated 2 h after soman (SOM 426
KET2h/AS) was comparable to that of mice not receiving any 427
treatment (SOM SAL) at 24 h (-17%) but with a better 428
recovery (-7% at day 7). 429

430 Brain Histology

431 In the SOM SAL group, HP-stained sections of PIR and AMG 431
showed numerous eosinophilic cells 24 h (92 ± 12 and $200 \pm$ 432
 12 stained cells per micrograph, respectively) and 72 h 433
postchallenge (83 ± 17 and 194 ± 29 stained cells per 434
micrograph, respectively) (Figure 2A,B). At day 7, the number 435

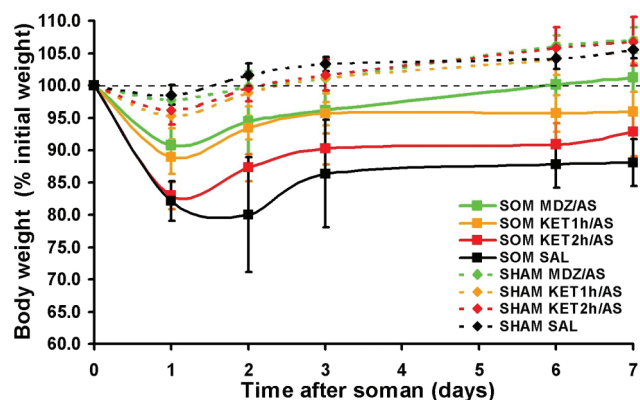


Figure 1. Evolution of the body weight of mice, either challenged with soman (SOM) or saline (SHAM), and then receiving one of the four treatments: saline (SAL), MDZ/AS, KET1h/AS, or KET2h/AS. Mice used for the HRMAS experiments are presented. For each group and at each time, results are expressed as the mean \pm SEM of the percentage of the initial body weight ($n = 28$ in each group at the beginning of the experiment; 7 mice were then taken out at each analysis time). At each time, all points of SOM groups were significantly different from the SHAM SAL group ($p < 0.001$) except at 6 and 7 days for the SOM MDZ/AS group.

(SHAM MDZ/AS: -14% , SHAM KET1h/AS: -14% and SHAM KET2h/AS: -18%).

Effect of Soman and Soman-Induced Seizures (SOM SAL vs SHAM SAL). In the PIR/AMG, 13 metabolites significantly varied in the SOM SAL group as compared to the SHAM SAL group, in accordance with our previous work.²⁷ We focused in this paper on 8 of them that could be more easily related to pathophysiological events, since they showed the most important changes and received the highest VIP values (see next paragraph, multivariate statistical analysis): Lac, lactate; Gln, glutamine; Ala, alanine; Ace, acetate; GPC, glycerophosphocholine; NAA, *N*-acetylaspartate; M-ins, myo-inositol; and Tau, taurine. The other metabolites were presented and discussed in our previous paper.²⁷

A long-lasting increase of Lac was noted from 24 h to day 7 postsoman, with a maximal increase ($+55\%$) after 72 h. Although decreasing thereafter, Lac level remained well above that measured in the SHAM SAL group (Figure 3A). Gln (Figure 3B) significantly increased at 24 h and reached a maximal elevation at 48 h ($+36\%$). It then decreased to reach an almost normal value at day 7. An increase of Ala (Figure 3C) was noted as soon as 4 h post soman, peaking at 48 h ($+107\%$), and decreasing thereafter to the control level at day 7. A significant but transient increase ($+95\%$) of Ace occurred, appearing only 4 h postchallenge (Figure 3D). An increase of GPC (Figure 3E) was also detected, reaching a plateau at 24 h (about $+40\%$) until 72 h, before complete normalization at day 7. NAA levels (Figure 3F) apparently decreased over time with the lowest level being recorded at 72 h (-43%) and were significantly different from the nonpoisoned animals. Despite a tendency to reincrease afterward, NAA level was still far from a complete normalization at day 7 (-27%). As for NAA, M-ins (Figure 3G) also decreased, and the lowest level was observed from 24 to 72 h (about -30 to 35%) before complete normalization at day 7. Finally, Tau (Figure 3H) slightly, but continuously, tended to decrease over the duration of the experiment, with the drop reaching statistical significance only at day 7 (-14%).

The CER of poisoned mice showed only limited changes compared to the SHAM SAL group: a significant decreases of NAA (-15%) at 72 h (Figure 4C) and of M-ins (-13%) at 24 h (Figure 4D).

Effect of the Nature and Timing of Administration of the Anticonvulsant Treatments (SOM MDZ/AS, SOM KET1h/AS, SOM KET2h/AS vs SOM SAL and SHAM SAL). *Effects of an Immediate Seizure Arrest (SOM MDZ/AS Group).* In the PIR/AMG, the immediate therapy prevented or reversed all the metabolic disturbances observed in poisoned animals. In the CER, the very limited soman-induced changes were also abated.

Effects of a 1 h Delay in KET/AS Treatment Initiation. KET/AS treatment, given 1 h after soman poisoning, could also normalize soman-induced changes of 5 out of the 8 metabolites of interest (Lac, Gln, Ace, M-ins and Tau) in PIR/AMG. Conversely, the protection afforded by KET1h/AS appeared incomplete for the other three metabolites, viz. Ala, GPC and NAA. For Ala (Figure 4B), a complete normalization was obtained at 4 and 24 h. However, at 48 h ($+43\%$) and 72 h ($+48\%$), although lower than in the SOM SAL group, the level remained above the control values observed in the SHAM SAL group, with the difference being significant only at 72 h. For GPC (Figure 3E), although the significant increase at 24 h postchallenge remained, the delayed treatment appeared to

of these damaged cells either continued to increase (PIR: 161 ± 51 stained cells) or tended to decrease (AMG: 94 ± 26 stained cells). An astrocytic activation, already important at 72 h, and even stronger at day 7, was detected in AMG. In PIR, the astrocytic activation was comparatively delayed, appearing only at day 7. In the CER neither damaged cells nor astrocytic activation were observed (Figure 2A,B).

When seizures were prevented or immediately stopped (MDZ/AS group), PIR and AMG were devoid of any HP-detectable brain damage or of any astrocytic activation (Figure 2A,B).

Compared to the SOM SAL group, the number of eosinophilic cells in the PIR and AMG from the KET1h/AS and KET2h/AS groups, although showing grossly similar temporal profiles, was either considerably reduced (KET1h/AS: 70 – 80% decrease of the number of stained cells) or moderately reduced (KET2h/AS: 30 – 45% decrease of the number of stained cells) for any of the time points. GFAP immunoreactivity in sections of the KET1h/AS and KET2h/AS groups, 72 h postchallenge (important in the AMG; almost negligible in the PIR), were similar to that seen in the SOM SAL group at the same time point and location. However, compared to the important GFAP immunoreactivity seen at day 7 in the SOM SAL group, KET/AS treatment had a clear impact and reduced the GFAP immunoreactivity area: -70 to -80 and -40% in the KET1h/AS and KET2h/AS groups respectively.

In all groups, CER was devoid of any apparent damaged cells and of astrocytic activation.

Temporal Profile of the Metabolite Changes

Effect of the Treatment Paradigms in Nonpoisoned Mice. Compared to mice of the SHAM SAL group, none of the anticonvulsant treatments appeared to induce any long-lasting changes in brain metabolite levels in PIR/AMG or CER. In Figure 3 and 4, only the SHAM SAL group is thus presented. However, at 4 h, during sedation, there was a significant decrease of Glu in PIR/AMG in the three treatment groups

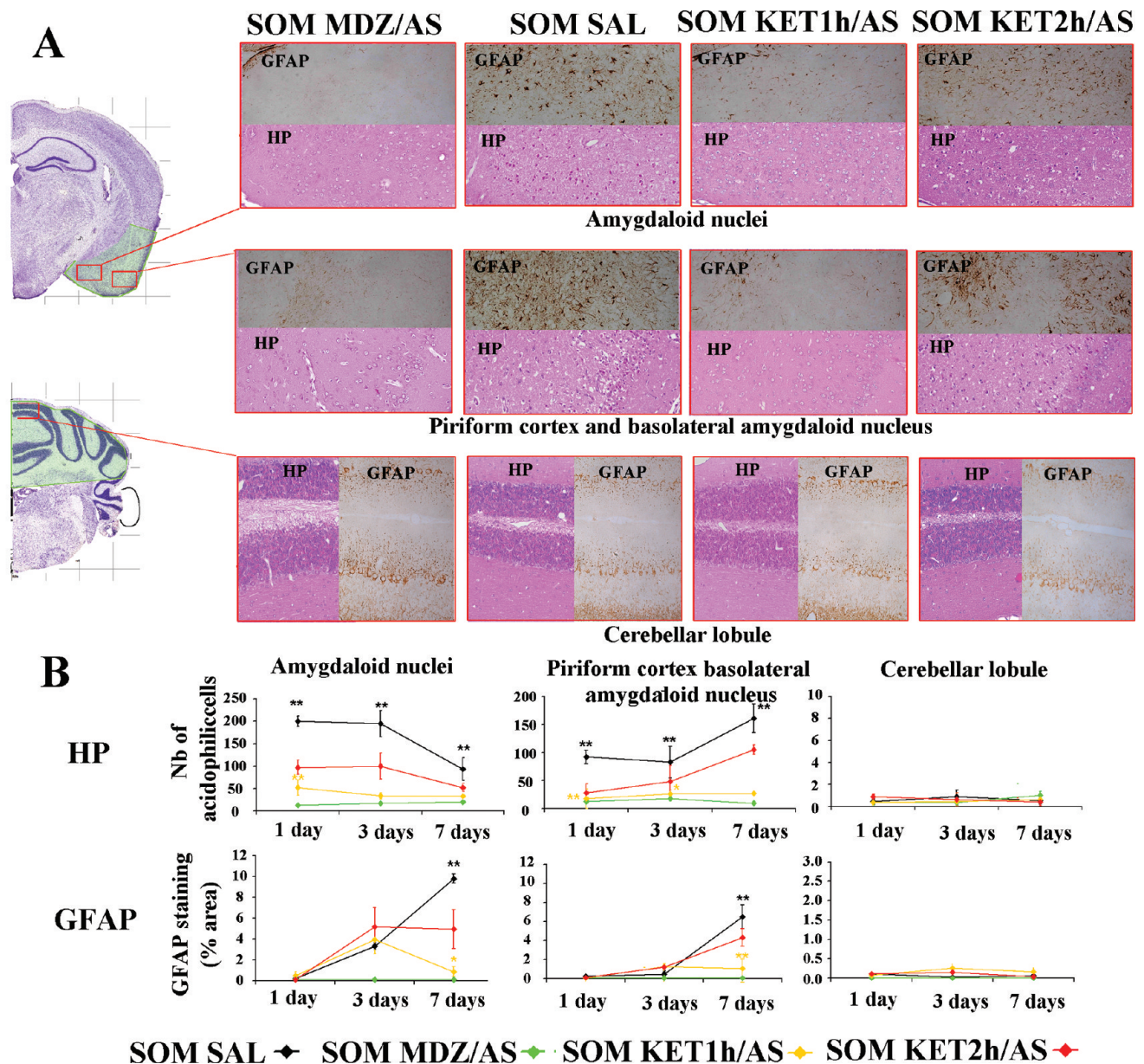


Figure 2. (A) Plates of coronal sections taken from Paxinos and Franklin mouse brain atlas.⁵⁴ The red rectangles are the areas chosen to acquire the micrographs. Examples of micrographs 3 days (HP) or 7 days (GFAP) postchallenge from SOM SAL, SOM MDZ/AS, SOM KET1h/AS, and SOM KET2h/AS groups. The green zones on the coronal diagrams are the locations of the biopsies used in the HRMAS study. (B) Quantitative histology. On the HP-stained sections, the number of eosinophilic cells per microphotograph was counted and expressed as the mean \pm SEM ($n = 7$ for each time and each group). On the sections processed for GFAP immunohistochemistry, the percentage of the microphotograph area occupied by reactive astrocytes was measured and expressed as the mean value \pm SEM ($n = 7$ for each time and each group). The diagrams show the results obtained in the amygdala, piriform cortex, and cerebellum of the SOM SAL (black curves), MDZ/AS (green curves), KET1h/AS (orange curves), and KET2h/AS (red curves) groups. Groups were statistically compared by an ANOVA per ranks followed by a posthoc test: black ** $p < 0.01$ between SOM SAL and SOM MDZ/AS groups, and orange ** $p < 0.01$, * $p < 0.05$ between SOM KET1h/AS and SOM SAL groups.

536 progressively normalize GPC levels (+22 and +16% at 48 and
537 72 h in the SOM KET1h/AS group vs +39 and +38% at the
538 same time points in the SOM SAL group). For NAA, a
539 decrease was recorded in the SOM KET1h/AS group with,
540 compared to the SOM SAL group, similar temporal profile but
541 with reduced values (maximal change: -13% at 72 h in the
542 SOM KET1h/AS group vs -43% in the SOM SAL group). In
543 the CER, the very limited soman-induced changes were no
544 more detectable.

545 *Effects of a 2 h Delay in KET/AS Treatment Initiation.* In
546 PIR/AMG, when the initiation of treatment was further

547 delayed, only one metabolite, Ace, showed a return to control
548 levels (Figure 3A). For GPC, no difference appeared with
549 KET1h/AS group. For the six other metabolites (Lac, M-ins,
550 NAA, Gln, Ala, Tau), they grossly followed the same temporal
551 profiles as those observed in the group of nontreated animals
552 (Figure 3). However, although the amplitude of variation
553 (decrease or increase depending on the metabolite) was
554 reduced, the metabolite levels never significantly differed from
555 those of the SOM SAL group and often remained significantly
556 different from those of the SHAM SAL group. As for the other

Piriform cortex and Amygdala

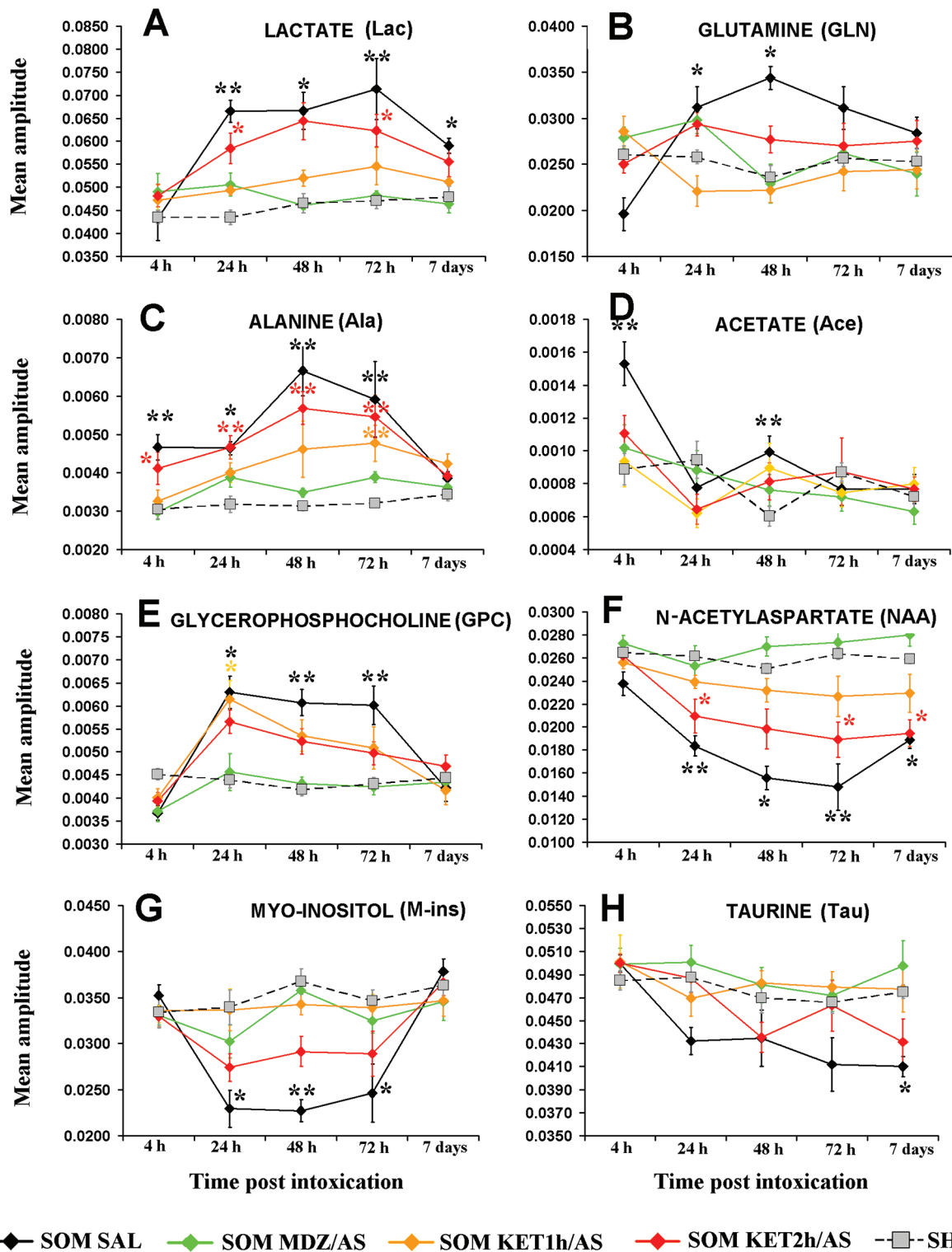


Figure 3. Evolution of mean metabolite amplitudes \pm SEM from 4 h to 7 days after soman intoxication in PIR and AMG for the four SOM groups (solid line and full diamond) and the SHAM SAL group (dashed line and gray open square). Black, saline treatment (SAL); green, MDZ/AS treatment; orange, KET1h/AS treatment; red, KET2h/AS treatment. Groups were statistically compared by an ANOVA per ranks followed by a posthoc test: black ** $p < 0.01$, * $p < 0.05$ between SOM SAL and SHAM SAL groups; green ** $p < 0.01$, * $p < 0.05$ between SOM MDZ/AS and SHAM SAL; orange ** $p < 0.01$, * $p < 0.05$ between SOM KET1h/AS and SHAM SAL groups; and red ** $p < 0.01$, * $p < 0.05$ between SOM KET2h/AS and SHAM SAL groups. For the sake of clarity, all the statistical differences with SOM SAL group are omitted.

Cerebellum

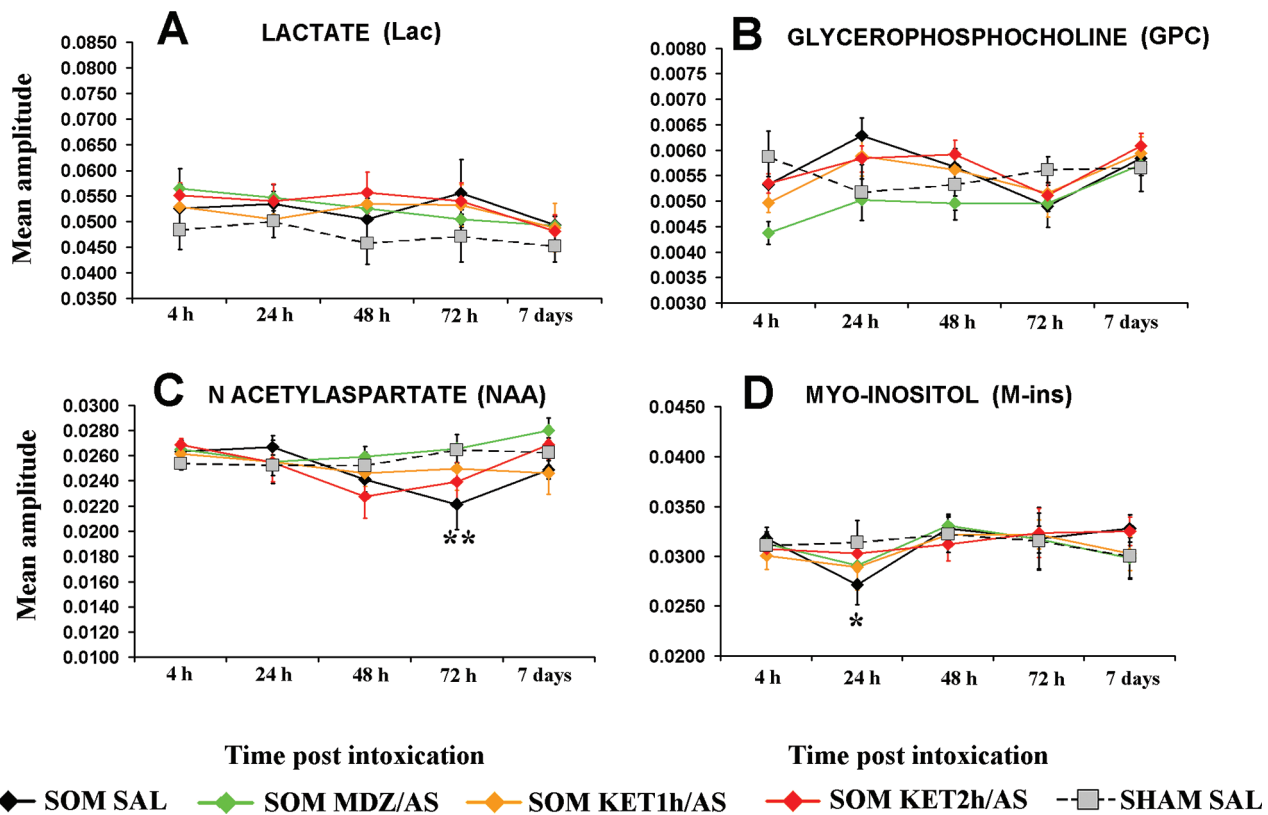


Figure 4. Evolution of mean metabolite amplitudes \pm SEM from 4 h to 7 days after soman intoxication in CER for the four SOM groups (solid line and full diamond) and the SHAM SAL group (dashed line and gray open square). Black, saline treatment (SAL); green, MDZ/AS treatment; orange, KET1h/AS treatment; red, KET2h/AS treatment. Groups were statistically compared by an ANOVA per ranks followed by a posthoc test: black ** $p < 0.01$, * $p < 0.05$ between SOM SAL and SHAM SAL groups.

557 two treatment paradigms, the soman-induced changes in
558 metabolite levels in CER were no longer detected.

559 Multivariate Statistical Analysis

560 **Statistical Model of the Soman-Induced Metabolic**
561 **Changes in PIR/AMG ("Soman Model").** The PCA analysis
562 (data not shown) allowed us to identify four groups among the
563 102 observations ($n = 57$ from the present study and $n = 45$
564 from the previous study²⁷). These groups were considered as Y
565 classes for the PLS-DA analysis. They are labeled as follows:
566 SHAM SAL for the class containing all nonintoxicated animals,
567 SOM SAL 4 h for the class of animals euthanized 4 h post
568 soman, SOM SAL 24–72 h for the class containing animals
569 euthanized 24, 48, and 72 h after soman and finally SOM SAL 7
570 d for the group containing animals euthanized at day 7 after
571 soman.

572 The observation of the score plot of our PLS-DA model
573 provided a global view of all metabolic events. First, it showed a
574 clear separation between SHAM SAL and SOM SAL classes
575 (Figure 5A). Second, a hypothetical evolution pathway over
576 time was suggested starting from SHAM SAL class and ending
577 to SOM SAL 7 d class. Finally, it can be observed that SOM
578 SAL 24–72 h and SHAM SAL classes are opposed relative to
579 the first component axis, and that SOM SAL 7 d class is close to
580 the SHAM SAL class.

581 **Validation of the Model.** Our PLS-DA model was built
582 with three components and was characterized by a good fit
583 ($R^2Y = 0.64$) and predictability ($Q^2 = 0.62$). As shown by

permutation tests, no permuted value outperformed the 584
original values either for R^2Y or Q^2 . The Q^2 -intercept was 585
clearly negative, evidencing the absence of any overfit of the 586
model for SHAM SAL (Figure 6A), SOM SAL 4 h, and SOM 587
SAL 24–72 h classes. For SOM SAL 7 d group, some R^2Y and 588
 Q^2 values of permuted models were equivalent or greater than 589
the original one (not shown). However, the CV-ANOVA test 590
indicated highly significant ($p < 10^{-6}$) differences between 591
classes. 592

Eight metabolites (variables) had a VIP > 1 (NAA, Ace, M- 593
Ins, GPC, Lac, Ala, Gln and Tau), indicating their most 594
important contribution in discriminating metabolic profiles 595
between the four classes (Figure 6B). Moreover, the 596
importance of variables in each class could be deduced from 597
the observation of the loading scatter plot (Figure 6C). Two 598
groups of variables were opposed along the first component: 599
GPC, Lac and Gln on the left, associated with SOM SAL 24– 600
72 h class and M-ins, NAA on the right, associated with SHAM 601
SAL class. This indicates that variables in the first group were at 602
their highest level in the SOM SAL 24–72 h class and that 603
variables in the second group were at their lowest level in this 604
class. Figure 6C also shows that Ace was at its highest level in 605
the SOM SAL 4 h class. 606

Prediction of Treatment Efficacy Using the "Soman 607
Model". As the statistical model described above presented a 608
relatively good predictive power ($Q^2 = 0.6$), it was used to 609
predict the efficacy of treatments (drug combinations and 610

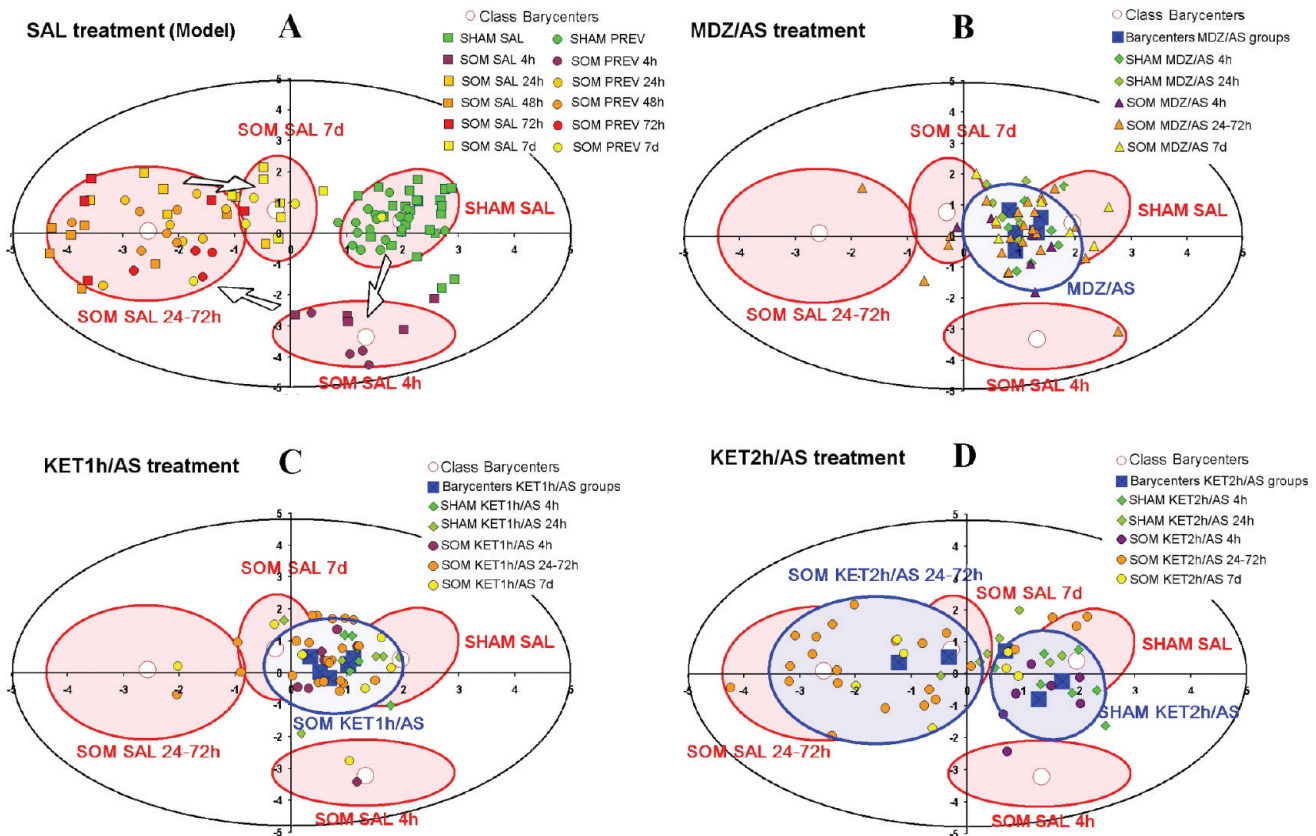


Figure 5. (A) “Soman model”: First two components score plot of the PLS-DA model obtained using the quantitated metabolite amplitudes from PIR/AMG of SHAM SAL group ($n = 25$), SHAM from the previous (PREV) experiment ($n = 18$), SOM SAL group ($n = 32$), and SOM from previous experiment ($n = 27$). The red ellipses were drawn around observations of the four classes: “SHAM SAL” (green), “SOM SAL 4 h” (purple), “SOM SAL 24–72 h” (red and orange), and “SOM SAL 7 d” (yellow). The separation between classes is clearly observed. A hypothetical evolution pathway over time is suggested by the arrows. (B, C, D) Projection of treated animals, whether intoxicated or not (blue ellipses); the red ellipses represent the four classes of the “soman model” described in A.

611 timing of administration), considering that animals treated with
612 the most efficient paradigm should be classified in the SHAM
613 SAL group.

614 Score plot projections (Figure 5) and the classification table
615 (Table 1) well synthesized the individual metabolic profiles and
616 their normalization by treatments.

617 As expected, all animals of the SHAM MDZ/AS (Figure 5B),
618 SHAM KET1h/AS (Figure 5C), SHAM KET2h/AS (Figure
619 5D) groups appeared in the immediate vicinity of the SHAM
620 SAL class and had a high classification rate of 91–92% (Table
621 1). This well depicts the limited impact of the considered
622 treatment paradigms on brain metabolism in nonintoxicated
623 mice.

624 Concerning SOM groups, both the SOM MDZ/AS and
625 SOM KET1h/AS mice were also projected close to the SHAM
626 SAL class (Figure 5B,C), with more than respectively 84 and
627 76% of animals that could fit in this class (Table 1). In contrast,
628 SOM KET2h/AS animals clearly differed from those of SHAM
629 SAL (Figure 5D), and less than 35% of them were classified in
630 the SHAM SAL class, while more than 50% had a metabolic
631 profile of the SOM/SAL 24–72 h class (Table 1). This
632 indicates that the delayed administration of KET/AS, 2 h
633 postchallenge, appeared poorly effective to counteract soman-
634 induced metabolism disturbances in the brain.

635 We can note that 4 h after intoxication, in the PLS-DA
636 “soman model”, very few animals (2/21) from MDZ/AS,
637 KET1h/AS or KET2h/AS groups were found in the SOM SAL

4 h zone. At this time, all these animals were sedated or just out
638 of anesthesia. This tends to confirm that the SOM SAL 4 h
639 zone can be considered as a “seizure zone”, a metabolic state
640 specific to animals with long lasting seizures.
641

642 In the CER, no group appeared in the PCA analysis of
643 metabolite concentrations. If the classes identified with PIR/
644 AMG were used for the PLS-DA analysis of CER, no validated
645 model could be found.

646 ■ DISCUSSION

647 Using several approaches (clinical symptoms, quantitative brain
648 histology and HRMAS NMR metabolic analysis), the present
649 study was undertaken to increase the confidence in our
650 previously published model²⁷ and to evaluate the impact of
651 three treatment paradigms on the soman-induced brain
652 metabolic changes: one immediate therapy known to be
653 efficient (MDZ/AS) and two delayed therapies based on the
654 combination of KET and AS. We tried to correlate the
655 metabolic changes measured by proton HRMAS NMR to
656 histopathological and clinical events.

657 Effect of Soman in the Absence of Any Treatment

658 **Clinical Symptoms.** All mice of the SOM SAL group
659 (when they survived) showed, among other severe symptoms,
660 long-lasting convulsions and drastic and durable loss of their
661 body weight.

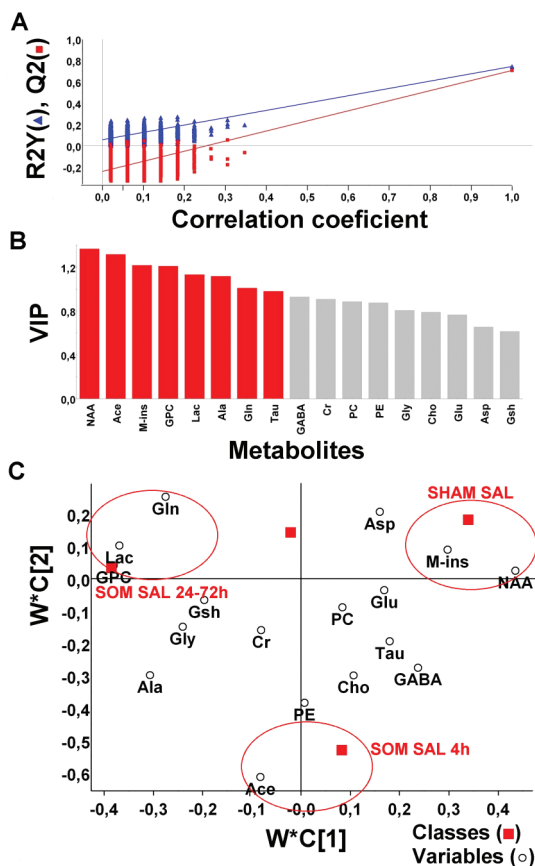


Figure 6. (A) Validation of the model for SHAM SAL class, using 999 permutations across three components. The regression line represents the correlation coefficient between the original and permuted Y-variables against R^2Y (green) and Q^2 (blue). (B) VIP plot: In red are represented the metabolites with a VIP > 1. (C) Loading plot of the model, showing variables and classes. Red ellipses regroup the most determinant metabolites (variables) associated with each class.

Table 1. Classification of Animals of the Treated Groups Using the PLS-DA “Soman Model”^a

groups of treatment	classes of the “soman model”			
	SHAM SAL	SOM SAL 4 h	SOM SAL 24–72 h	SOM SAL 7 d
SHAM MDZ/AS	32/35 (91.4%)		1/35	2/35
SHAM KET1h/AS	32/35 (91.4%)	2/35		1/35
SHAM KET2h/AS	34/37 (91.9%)		3/37	1/37
SOM MDZ/AS	31/37 (83.8%)	2/37	3/37	1/37
SOM KET1h/AS	28/37 (75.7%)	2/37	5/37	2/37
SOM KET2h/AS	14/41 (34.1%)	1/41	22/41	4/41

^aThe rate of classification in the SHAM SAL group (in brackets) is considered as a criterion for the evaluation of treatment efficacy.

presence/disappearance of actual electrographic seizure activity. 665 This assertion is based on (i) the available bibliographic data 666 (references in the Introduction section and in the following 667 paragraphs) and (ii) our measurement of the body weight loss 668 that is a recognized indicator of soman-induced brain 669 damage^{9,41,53,59} and thus of long-lasting seizure activity owing 670 to the relationship between the two events.^{5,53,60,61} Although 671 during MDZ or KET sedation, the absence of convulsions 672 cannot be only interpreted as the termination of seizures, the 673 prevention of brain damage is a good indicator of their action 674 on seizures. 675

Histology and Metabolism. PIR and AMG, two brain 676 areas known to be extremely sensitive to soman brain damaging 677 effects,^{4–7} expectedly showed profound structural changes as 678 evidenced by the presence of numerous eosinophilic cells. 679

In the AMG, the number of damaged cells, as evidenced by 680 HP staining, peaked during the first three days before declining. 681 Such observation is also similar to the results obtained by 682 others in soman-intoxicated rats and mice;⁷ this decrease of 683 damaged cells, after the third day, is mostly caused by the 684 disappearance of the damaged cells rather than by their 685 recovery,⁷ although this can only be ascertained by a precise cell 686 counting. In the same brain area, no astrocyte activation was 687 discernible at 24 h. In literature, the delay for the induction of 688 GFAP after soman poisoning is not clearly established. Indeed, 689 some works indicated a rapid activation of astroglia within the 690 very first hours following challenge, as evidenced by the 691 increase in GFAP mRNA^{62,63} or even GFAP immunoreactivity.¹⁴ 692 In this last work, however, the animals had been operated 693 a week before soman poisoning, and this may well have induced 694 an astroglial activation. On the other hand, our results are well 695 coherent with those of others evidencing a weak or absent 696 GFAP immunoreactivity in the AMG or PIR before 2–3 days, 697 and an important increase of GFAP immunoreactivity at day 7 698 after soman exposure.^{4,13} 699

In the PIR, the number of eosinophilic cells at 24–72 h was 700 less important than in the AMG at the same time points but, 701 instead of further decreasing, continued to increase until day 7. 702 As revealed by GFAP immunohistochemistry, astrocyte 703 activation was weak the third day of the intoxication and 704 strongly increased at day 7. All these results suggest that 705 structural damage and accompanying astrogliosis in the PIR 706 were apparently delayed compared to the AMG. To our 707 knowledge, such a phenomenon has never been reported so far. 708

Soman-induced changes on PIR and AMG metabolism were 709 not distinguished in this study, but globally, a parallelism with 710 the histological data was observed. The Lac level seemed to 711 well fit with the number of acidophilic cells (HP staining) in 712 AMG, with a large increase from 24 to 72 h and a partial 713 recovery at day 7. It is tempting to link these two perturbations 714 since lactate has been reported to be involved in tissular 715 acidosis.⁶⁴ However, this was not fully confirmed in PIR, where 716 acidophilic staining was increased at day 7 but not followed by 717 an increase of the Lac level. 718

The NAA decrease closely followed the inverse kinetics of 719 Lac. A decrease of this metabolite has already been reported to 720 be associated with a deficit in mitochondrial activity³² but it 721 may also be related to neuronal death.⁶⁵ Extending the duration 722 of the study beyond 7 days might help to know the relative 723 contribution of these two events. 724

At day 7, the astrocyte activation was high in AMG and PIR, 725 while most of the metabolites recovered their control level 726 except Lac, NAA and Tau. The Tau level slowly decreased after 727

662 Despite the lack of EEG recording in the present study, our 663 clinical evaluation of the absence/presence/disappearance of 664 convulsions can be trustfully interpreted in terms of absence/

728 24 h post soman, and this decrease became significant at day 7.
729 In a mouse model of kainate-induced SE, an increase of Tau
730 rather than a decrease was observed in the hippocampus at day
731 7.⁶⁶ More in the line of our data are the reports of antiepileptic
732 effects of taurine treatments owing to the effects of Tau on
733 neuronal excitability and on the modulation of recurrent seizure
734 activity.^{67,68} Tau was also studied in a model of long-term
735 effects of kainate-induced seizures in rats (6 months) of less
736 relevance to our study.³⁵ However a decrease in AMG/PIR was
737 also reported. Therefore, a more focused study would be
738 necessary to test if the decrease in Tau levels we observed may
739 have functional consequences and facilitate recurrent seizures.
740 At difference to AMG/PIR, the CER appeared free of any
741 detectable damage (HP staining) and astrocytic activation. This
742 observation is well in line with that of others showing that, at
743 least in soman poisoned rodents, damage to this structure are
744 either rare and limited to the vermis^{5,6} or even absent.⁷ CER
745 also presented very little changes in metabolism confirming our
746 previous results.²⁷ Only NAA significantly decreased 72 h after
747 intoxication with a recovery at day 7. To the best of our
748 knowledge, data on OP-induced changes in CER are limited.
749 We reported some inflammatory gene response changes in a
750 comparable mouse model.¹⁶ Some more delayed modifications
751 of cellular signaling have also been reported.⁶⁹ All in all, this
752 clearly shows that CER cannot be considered as devoid of any
753 changes.

754 **PLS-DA "Soman Model".** The PLS-DA model of soman-
755 induced metabolic changes, built with HRMAS NMR metabolic
756 data, provides a synthetic view of all the metabolic events
757 occurring during the soman intoxication. It is in accordance
758 with our previous work,²⁷ even if slight differences are observed
759 in this study for some metabolites. The score plot and the
760 loading plot show that there is a good agreement between the
761 classification of metabolic profiles and the clinical observations,
762 body weight or histopathology. More specifically, the first
763 component of the model clearly separates SHAM SAL and
764 SOM SAL 24–72 h classes. The latter corresponds to the
765 period when brain lesions, cerebral edema and neuro-
766 inflammation become more pronounced. It appears like a
767 "neuropathological zone", with high levels of GPC, Lac and
768 Gln, and low levels of NAA and M-ins. The second component
769 mainly separates SOM SAL 4 h class from the others. This class
770 is characterized by a high level of Ace, a metabolite that is not
771 abundant in normal brain. Since animals have fully developed
772 epileptic activity at that time point, this graphic zone could be
773 considered as the "seizure zone". The third component allows
774 separating SOM SAL 7 d from the other classes. A
775 normalization of most of the metabolites is observed at this
776 time point, and the difference with SHAM SAL mice is mainly
777 due to NAA and Tau levels, which remain slightly decreased.
778 This class regroups mice that are recovering.

779 In a second step, we thus challenged our model with the
780 results obtained after treatment with anticonvulsant combina-
781 tions.

782 Effect of Treatments on Soman-Intoxicated Mice

783 **MDZ/AS Treatment.** An immediate treatment with a
784 MDZ/AS combination prevented the appearance of con-
785 vulsions and clearly the loss in body weight of the animals. This
786 was paralleled by a good histological neuroprotection (neither
787 detectable brain damage nor astrocyte activation) as well as by a
788 good protection against the soman-induced metabolic changes,
789 both in CER and in PIR/AMG. Furthermore, using the PLS-

DA "soman model", it appeared as the most efficient treatment,
790 with 84% of observations classified in the SHAM SAL group.
791 From a methodological point of view, these mice constituted a
792 very good positive control for the evaluation of the efficacy of
793 the other treatments. This confirms that MDZ/AS, when
794 administered within minutes after soman intoxication, is an
795 efficient antidote against the central effects of the poisoning and
796 is capable to prevent seizures and related brain dam-
797 age.^{40,53,70,71} 798

KET1h/AS Treatment. During SE, benzodiazepines are
799 usually ineffective, and combinations of KET have proven their
800 efficacy in different animal models such as guinea pigs⁵¹ as well
801 as mice.¹⁵ Administration of KET/AS 1 h postchallenge
802 produced almost an immediate arrest of ongoing convulsions.
803 Without EEG recording, it is not possible to ascertain that the
804 seizures were also abated but the loss of body weight being
805 limited, this is highly probable. Although the recovery in body
806 weight at day 7 was less pronounced than after an immediate
807 treatment with MDZ/AS, the body weight was not significantly
808 different from that of the SHAM groups. Administration of
809 KET/AS in poisoned animals also did not prove more
810 deleterious than in nonpoisoned animals (death rate 7 and
811 8% respectively), showing that at an appropriate dosage, KET
812 can be used in poisoned individuals. Like in Dhote et al.,¹⁵ the
813 paradigms were here chosen to be proofs of concept. In
814 histological sections, the number of eosinophilic cells in
815 sensitive brain areas (AMG, PIR) was considerably reduced
816 compared to that in the SOM SAL group. Moreover, the
817 treatment strongly reduced most of the soman-induced
818 metabolic changes in PIR/AMG or CER. Using the PLS-DA
819 "soman model" (for PIR/AMG), 76% of the observations were
820 classified in the SHAM SAL group. All these observations are
821 thus consistent with our previous data on KET/AS
822 combinations.^{15,51,52} In these previous papers, we also
823 mentioned that the neuroprotection was not complete. Here
824 also, some perturbations could still be observed in PIR/AMG
825 of the treated animals, e.g., in the levels of Ala, NAA and GPC,
826 or in astrogliosis. Indeed, an important astrocytic activation was
827 still evidenced at 72 h with a magnitude comparable to that
828 detected at the same time in the SOM SAL group. Conversely,
829 at day 7, KET/AS treatment could almost totally reduce GFAP
830 immunoreactivity to the level of controls. Very comparable
831 results were obtained at 48 h and 7 days with slightly different
832 KET/AS paradigms in a comparable mouse model.¹⁵ That
833 seems to indicate that KET/AS, maybe because of the delay in
834 administration, could not prevent the initiation of astrogliosis
835 but markedly reduced its duration. Interestingly, a similar effect
836 of KET/AS was observed on the GPC levels: while KET/AS
837 could not prevent its increase at 24 h, the treatment apparently
838 contributed to a faster return to control level. 839

Astrogliosis may be initiated by pro-inflammatory cytokines
840 even in the absence of cellular damage.^{72–74} We previously
841 hypothesized that the GPC increase may also be related to the
842 activation of different pro-inflammatory enzymes.²⁷ KET/AS
843 clearly counteracted soman-induced neuroinflammation 48 h
844 and 7 days postchallenge,¹⁵ but data are lacking regarding the
845 possible effects of these combinations on the early pro-
846 inflammatory signals. 847

Globally, a delayed administration of KET/AS could, for
848 many of the parameters, be as effective as an immediate
849 treatment with MDZ/AZ. However, 1 h of fully developed
850 seizures before KET/AS administration had an obvious impact,
851 and in the PLS-DA "soman model", the KET1h/AS mice were 852

853 projected near the SHAM SAL zone but were more scattered
854 than the MDZ/AS mice, and a few points even appeared in the
855 SOM SAL 24–72 h class.

856 **KET2h/AS Treatment.** By further delaying the initiation of
857 treatment with KET/AS to 2 h postchallenge, a reduced
858 neuroprotection was anticipated as described earlier.^{51,52} This
859 was fully confirmed in the present study. The delayed
860 administration of KET/AS only transiently stopped the
861 convulsions in spite of repeated injections. These long-lasting
862 seizures had a clear impact on the body weight loss that was
863 comparable to that of untreated mice. The neuroprotection was
864 indeed partial, with numerous eosinophilic cells and an
865 extended astrogliosis still present although less prominent
866 than that observed in untreated poisoned animals. The
867 metabolic perturbations induced by the administration of
868 soman were observed, but they were slightly reduced by the
869 treatment. As a consequence, in the score plot of the PLS-DA
870 “soman model”, the KET2h/AS treated mice were largely
871 scattered, with many observations in the SOM SAL 24–72 h
872 zone, reflecting the low efficacy of the treatment on the changes
873 in brain metabolism.

874 ■ CONCLUSION

875 Using proton HRMAS NMR of mouse brain biopsies, we have
876 established a statistical model that synthesizes all the metabolic
877 events occurring in some areas of the brain during 7 days after
878 soman intoxication. In this model, “control” and “pathological
879 zones” are clearly separated. Moreover, classification with this
880 model is robust, and the efficiency of neuroprotective
881 treatments can be globally evaluated by the classification of
882 treated animals in the different zones. Our results show that
883 animals that follow the entire “soman model” pathway or that
884 enter in the “pathological zone” will suffer from brain
885 consequences of soman intoxication.

886 Analysis of the individual variations of each metabolite,
887 associated with a histological approach, could help to better
888 understand the complex pathophysiological events occurring
889 during the intoxication and may provide leads to elaborate
890 further treatments. Further experiments should also focus on
891 the changes in metabolism occurring beyond 7 days, e.g., in
892 order to confirm or not the recovery of NAA, Lac or Tau. The
893 hypothetical link between GPC and inflammation has to be
894 more precisely investigated. Metabolic profiling using HRMAS
895 NMR data thus provides an interesting diagnostic and
896 predictive tool for the experimental evaluation of the cerebral
897 consequences of nerve agent poisoning and treatments.
898 Moreover, these findings could be very useful to improve the
899 potential of in vivo proton magnetic resonance spectroscopy as
900 a noninvasive diagnostic tool in humans.

901 Finally, the present work also supports further the
902 anticonvulsant and neuroprotective efficacy of a KET/AS
903 combination during refractory SE, its effect being comparable
904 but not entirely similar to that of an immediate treatment with
905 a benzodiazepine. Approximately 1 h of soman-induced seizures
906 still remains the limit of this treatment as demonstrated by the
907 poor neuroprotection afforded by KET/AS given 2 h
908 postchallenge. Extending further the delay before a given
909 treatment is especially important since such delays may be
910 expected because of confusion in battle or in the aftermath of
911 a terrorist attack, the two main circumstances where nerve agents
912 such as soman might be used.

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Notes

The authors declare no competing financial interest.

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