Endozepine stupor
Recurring stupor linked to endozepine-4 accumulation

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Summary
Recurring stupor can be caused by repeated metabolic, toxic or structural brain disturbances. Recently, cases of recurring stupor with fast EEG activity were shown to display increased endogenous benzodiazepine-like activity during the episodes of stupor. Patients with recurring stupor underwent extensive metabolic and toxicologic screening, EEG and brain imaging. Endozepines and exogenously administered benzodiazepines were assayed in plasma and CSF by means of mass spectrometry. Flumazenil, a benzodiazepine antagonist was administered and the behavioural and EEG responses monitored. Treatment with oral flumazenil was attempted in selected cases. Twenty patients were found with recurring stupor. Episodes had begun between ages 18 and 67 years, and in nine patients, had disappeared spontaneously after 4–6 years with symptoms. Stupor lasted hours or days. Onset of the episodes and frequency were unpredictable. Patients were normal between attacks. Stupor was characterized by initial drowsiness, staggering and behavioural changes, followed by deep sleep and spontaneous recovery with post-ictal amnesia. Biochemical screening and brain imaging were always normal. Ictal EEG showed fast background activity, and flumazenil transiently awoke the patients and normalized the EEG. In the nine cases examined, endozepine-4 levels were increased during the stupor. Oral flumazenil reduced the frequency of the attacks in three of these nine patients. Recurring episodes of stupor may be due to increased endozepine-4. We propose the term ‘endozepine stupor’ for such episodes. Endozepine-4 is an endogenous ligand for the benzodiazepine recognition site at the GABA receptor, with unknown molecular structure.

Keywords: stupor; coma; benzodiazepine; endozepine; flumazenil

Abbreviations: GABA = γ-aminobutyric acid; HPLC = high-pressure liquid chromatography; MMSE = Mini-Mental State Examination

Introduction
Recurrent episodes of stupor and coma may be due to recurrent epileptic seizures (Thomas et al., 1992), non-communicating hydrocephalus caused by a third ventricle cyst (Plum and Posner, 1980) or aqueductal stenosis (Galeotti et al., 1991), episodes of liver, kidney or respiratory failure (Plum and Posner, 1980) or exogenous administration of psychoactive drugs, in particular benzodiazepines (Plum and Posner, 1980). In recent years, we reported four patients with recurring episodes of stupor or coma characterized by fast EEG activity responsive to flumazenil, a benzodiazepine antagonist, in the absence of exogenous benzodiazepine administration (Montagna et al., 1990; Rothstein et al., 1992c; Tinuper et al., 1992, 1994). In these patients, the presence of an uncharacterized ligand to central benzodiazepine receptors in plasma and CSF during the ictal episodes implicated the involvment of the γ-aminobutyric acid (GABA)-ergic system (Rothstein et al., 1992c). Endozepine-4, an endogenous ligand for the benzodiazepine recognition site of the GABA receptor (Rothstein et al., 1992b), was markedly increased in plasma and CSF during the episodes of stupor (Rothstein et al., 1992b, c; Tinuper et al., 1994). Since the elevated plasma endozepine-4 levels observed during the episodes were similar to the concentrations of exogenous benzodiazepine needed to cause stupor in humans, the increase in endozepine-4 seemed to play a pathogenetic role in the causation of stupor.
Following our initial reports, we have observed 16 additional cases with recurring stupor displaying the features of this syndrome, which we originally termed idiopathic recurring stupor, and we have studied complete spontaneous episodes making serial measurements over time during the attack. We report here the clinical and laboratory features in this series of 20 patients, including our original ones, since we think that such cases constitute a novel not so rare syndrome, which we now prefer to term ‘endozepine stupor’.

Material and methods
Twenty patients referred to our Institute with a history of repeated stupor or coma episodes underwent a standard protocol during the period between attacks, and whenever they were admitted during the actual episodes of stupor or coma.

The interictal study comprised history-taking and physical and neurological examinations. Laboratory tests included complete blood count, serum sodium, potassium, chloride, calcium, phosphorus, bicarbonate, urea, creatinine, glucose, aspartate aminotransferase, alanine-aminotransferase, alkaline phosphatase, fasting and post-prandial arterial and venous ammonium, and plasma osmolality. Sixteen patients also underwent CSF examination for proteins, glucose and cells. Intercital plasma and CSF samples were obtained for endozepine-4 analysis (see below). All patients underwent 16-channel EEG studies utilizing the standard 21-electrode electrode system. In addition, 17 of them underwent 24-h polygraphic recordings including EEG, submental electromyography, ECG, breathing, and blood O₂ saturation monitored by ear oxymetry. These recordings were followed the day after by a multiple sleep latency test (Carskadon et al., 1986). Brain MRI or enhanced CT scans were performed in all patients. To exclude the possibility of transient episodes of stupor due to hepatic encephalopathy and portocaval shunts, even in the absence of any obvious indication of liver disease, three patients underwent liver biopsy, and 15 of them had abdominal ultrasound scans and Doppler studies.

Neurological evaluation and all the laboratory tests, with additional arterial gas-analytic determinations and urine and serum screening for opiates, ethanol, cocaine, benzodiazepines, barbiturates and tricyclic antidepressants, were again performed during the stuporous episodes in all 20 patients, monitored either by us (16 cases) or in other institutions (four patients) according to our protocol. All of the observed stuporous episodes were monitored by EEG, and the clinical and EEG effects of flumazenil administration (0.5–2 mg i.v. over 10 min) were tested. Ten patients underwent brain MRI or enhanced CT during the stupor. For nine of the total 20 patients, it was possible to determine the levels of endozepine-2 and endozepine-4 in blood and/or CSF during the stupor (see below). However, since the vast majority of the benzodiazepine activity migrated as endozepine-4 peaks, analysis was restricted to the latter. Blood and CSF samples were taken at the same time, as soon as the patient came under observation, and prior to flumazenil administration. In four of these patients, mass spectrometry of serum was performed in order to rule out the presence of synthetic benzodiazepine.

Endozepine-4 analysis
Serum and CSF samples were collected and stored at −70°C until analysis. One millilitre of serum or CSF was extracted with 1 ml of chloroform, the lower organic phase was then collected, dried by vacuum centrifugation, then reconstituted with 500 µl of distilled deionized water containing 0.1% trifluoroacetic acid (Rothstein et al., 1992c). Samples were then injected onto a high-pressure liquid chromatography (HPLC) 250×4 mm reversed phase column (Bio-SIL ODS 10, Bio-Rad) equilibrated with 0.1% trifluoroacetic acid in H₂O (Rothstein et al., 1992b). Samples were chromatographed by reverse phase HPLC as described previously (Rothstein et al., 1992b, c) to elute and separate the endozepines. Effluent was monitored by UV₂₅₉ nm and 1 min fractions were collected. Fractions were vacuum dried and reconstituted with distilled deionized water (250 µl) for endozepine-4 quantification. In some cases, fractions containing endozepine-4 activity were lyophilised, then reconstituted with a small volume (1–2 ml) of methanol: ethylacetate (1:1) and chromatographed by normal phase HPLC as described previously (Rothstein et al., 1992b, c). Using these HPLC methods, the only synthetic benzodiazepine that elutes within 4 min of endozepine-4 activity is lorazepam. Endozepine-4 was quantified in HPLC fractions by competitive displacement studies (Rothstein et al., 1992c). Aliquots of the reconstituted HPLC fractions (5–100 µl) from either serum or CSF extracts were tested for their ability to inhibit 1 nM [³H]flunitrazepam (specific activity 87 Ci/mmol, New England Nuclear, Boston, Mass., USA) binding to rat cerebellar membranes. The aliquots were incubated in 0.25 ml of 50 mM NaPO₄ (pH 7.4) which contained 5 µM GABA, 1 nM [³H]flunitrazepam, and rat cerebellar membranes (80 µg protein/ml) for 1 h at 4°C. The reaction was terminated by vacuum filtration through glass fibre filters using a cell harvester (Cambridge Technology, Watertown, Mass., USA). The filters were transferred to vials (Wheaton Omnivials, Millville, NJ, USA) with scintillation cocktail (Safety Solve, Research Products International, Ill., USA) and the radioactivity measured. The amount of competition for the radioligand was compared with standard competition curves generated with known amounts of diazepam. Data were expressed as diazepam equivalents, defined as the number of pmoles of diazepam required to inhibit [³H]flunitrazepam binding to rat cerebellar membranes to the same extent as endozepines. All samples were analysed in triplicate; intra-assay variability was <5%.

Mass spectrometric analysis
To rule out the presence of contaminating synthetic benzodiazepine in patients’ sera, extracts of sera from four
patients with elevated endozepine-4 activity were analysed by gas chromatography–mass spectrometry. In parallel, normal serum doped with 5 nmol lorazepam was extracted and analysed to determine instrumental sensitivity and response. Lorazepam was chosen because it is the only synthetic benzodiazepine that elutes from HPLC in the vicinity of endozepine-4. Chloroform extracts (2 ml) from 1 ml of serum were dried, re-suspended in 1 ml of chloroform and the clear supernatants transferred carefully by pipette to small vials and evaporated under a stream of nitrogen. Dried residues were dissolved in 8 µl methanol and 3 µl aliquots of each sample analysed by gas-chromatography–electron ionization mass spectrometry. Mass spectra were recorded from 50 to 350 Da, and recorded spectra of lorazepam extracted from serum that matched from standards with a chemical signal to noise ratio of >100. Concentrations of lorazepam of >0.5 nmol/ml serum would be readily detectable using this procedure; the concentration of endozepine-4 assayed by radioreceptor binding in the four patients’ sera collected during stupor analysed mass spectrometrically exceeded, in diazepam equivalents, 5 nmol/ml.

All of the interictal studies and flumazenil treatment were done only after informed consent from each patient.

Results

Patient demographics (Table 1)

Twenty patients from all parts of Italy (Fig. 1) representing various social groups and different work environments were found to have endozepine stupor. Sixteen were males. Age at observation ranged from 24 to 69 (mean 50.6) years. The stupor episodes had begun between 18 and 67 (mean 48.3) years of age. The duration of the episodes varied between 2 h and 5 days (see Table 1). In four cases, stupor always appeared after waking in the morning, in three it was after lunch or dinner, and in the remaining cases it could be at any other time of the day. The onset of the stupor episodes was not related to either normal activities (wake–sleep cycle, dietary habits, alcohol ingestion) or psychological factors (emotional stress). Patients could have fairly frequent episodes (>6 per year) or rare attacks (1–6 per year), and often alternated frequent and infrequent attacks (see Fig. 2). The longest interval between two stupor episodes was 1 year (Case 6). In nine patients (Cases 2–10) the episodes had disappeared spontaneously 2–6 years previously, after 1–6 years of recurring episodes.

None of the patients had a family history of recurring stupor. Four of them were moderate and nine mild alcohol drinkers, eight were heavy smokers (>15 cigarettes per day); three had a mild chronic obstructive pulmonary syndrome. At the time of onset of the stuporous episodes all patients were in good mental and physical condition, and none were receiving medical treatment. No patient had a previous history of psychiatric disorders.

Interictal examinations

General, neurological and EEG examinations, and blood and urine tests between episodes were always normal. Liver
biopsy was normal (in Cases 1, 9 and 11). Abdominal ultrasound scans and Doppler study excluded portocaval shunts in the 15 patients examined. Polygraphy showed normal sleep patterns in all cases; there was only some sleep fragmentation in one subject (Case 15), affected by restless legs syndrome and in three patients with obstructive sleep apnea syndrome (with an apnea–hypopnea index of $>10$). Snoring was recorded in another seven cases besides the three affected with obstructive sleep apnea syndrome, but apneas were sporadic and sleep structure normal. Sleep blood saturation $O_2$ remained normal in all cases. Daytime somnolence measured by multiple sleep latency test was normal ($>10$ min) in 17 and borderline (between 5 and 10 min) in three cases. Brain CT and MRI were unremarkable in all patients.

**Ictal examinations**

We observed at least one episode of stupor or coma in all patients. In 14 cases, more than one ictal episode of stupor was directly monitored. Actual stupor could be (or not) preceded for hours or even days by a feeling of fatigue and malaise and increasing somnolence, associated with mental and motor slowing. Speech became slurred, gait uncertain. After resting on a chair or bed, the patients would lapse into unconsciousness within a few minutes. On other occasions, however, unaware of their condition they would try to go out or even to drive their cars, on occasion causing an accident (Cases 11 and 12). If restrained, they would refuse to take heed and even become aggressive and violent. However, one subject (Case 13) became unusually docile and emotional in the prodromic phase. Most subjects just ‘fell asleep’ or slumped onto the floor. During the episodes of mild stupor the patients could be aroused by acoustic, tactile or painful stimuli, and even answer questions briefly before again losing consciousness. In some cases, stupor progressed to coma. Patients could not be awakened at all and painful stimuli provoked only some more or less purposeful movements. Muscle tone was reduced in all cases, tendon reflexes were weak and abolished in the deepest episodes. Pupils were miotic, but always reactive to light. Oculocephalic reflexes were lost in only one patient (Case 16) during an episode of deep coma. Breathing was always regular, sometimes accompanied by slight snoring. Recovery was spontaneous and the time taken to regain full consciousness was proportional to the duration and depth of stupor. On recovery, patients appeared initially drowsy, confused and amnesic. Speech was slurred, gait uncertain. Patients were unable to remember things or recognize places and people. They answered questions with short sentences and wrongly, and on some occasions, would confabulate when invited to give details. When consciousness was fully regained, they were alert and oriented, but remained unable to recall the episode and the events of the hours and few days preceding the episode. No patient reported any oneric mental activity during the episodes.

Blood and urine tests, and CSF specimens where taken, were unremarkable during all instances of stupor. Blood $O_2$ always remained normal (saturation of $O_2 >90\%$) even in those patients with some evidence of respiratory disease. Brain CT scan and MRI performed during the stupor were normal and similar to those observed interictally. In no case was there evidence of blood benzodiazepine, barbiturates, tricyclic antidepressants, ethanol, opiates or cocaine. Blood sugar levels remained similar to the interictal values. On EEG, a diffuse, low amplitude unreactive fast activity with a peak at 13–16 Hz characterized all of the episodes of mild and deep stupor (Fig. 3). Intravenous administration of flumazenil 0.5–2 mg under EEG monitoring was done in all of the patients. It always led to a transient reappearance of EEG alpha activity associated with a behavioral awakening or arousal; however, this lasted only 10–15 min. Representative excerpts of the EEG tracings between and during the episodes, and of the effects of flumazenil are shown in Fig. 3.

**Measurements of endozepine-4**

In the nine patients who underwent endozepine-4 analysis, serum (nine subjects) and CSF (four subjects) levels between attacks were not different from those in controls, but they rose as high as 20 850 pmol/ml during the stuporous episodes.
Table 2  Ictal and interictal levels of endozepine-4 in serum and CSF of nine patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Ictal endozepine-4 (pmol/ml)</th>
<th>Interictal endozepine-4 (pmol/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Serum CSF</td>
<td>Serum CSF</td>
</tr>
<tr>
<td>1</td>
<td>455 100</td>
<td>0 5</td>
</tr>
<tr>
<td>9</td>
<td>300 –</td>
<td>– 25</td>
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<tr>
<td>10</td>
<td>623 454</td>
<td>5 –</td>
</tr>
<tr>
<td>11</td>
<td>366 (122–610) 335</td>
<td>4 –</td>
</tr>
<tr>
<td>12</td>
<td>602 –</td>
<td>0 –</td>
</tr>
<tr>
<td>16</td>
<td>625 –</td>
<td>0 –</td>
</tr>
<tr>
<td>17</td>
<td>9500 (n = 29) (1590 ± 171)</td>
<td>5 –</td>
</tr>
<tr>
<td>19</td>
<td>3500 –</td>
<td>0 –</td>
</tr>
<tr>
<td>20</td>
<td>20850 (n = 23) (10067 ± 1508)</td>
<td>4100 0</td>
</tr>
</tbody>
</table>

In patients 11, 17 and 20, serum values shown are averages; the range or mean ± SE values are reported in parenthesis. Control values for serum and CSF were 2.5 ± 0.99 (n = 19) and 2.2 ± 0.8 pmol/ml (n = 6), respectively.

(Table 2). CSF levels were available in four patients and were generally lower than concomitant serum analysis. There was no evidence of synthetic benzodiazepines by mass spectrometry in the four patients that underwent endozepine-4 analysis.

In two patients (Cases 17 and 20), with frequent attacks, we were able to monitor the levels of serum endozepine-4 serially (every 4 h) on the days prior to and during a spontaneous episode of stupor. During and after the attack, Case 20 received serial EEG and neuropsychological tests consisting of the Mini-Mental State Examination (MMSE) (Folstein et al., 1975) performed every 12 h. As shown in Fig. 4, serum endozepine-4, absent in the interictal period, rose during the stupor and fell when the patient spontaneously regained consciousness 3.5 days later. MMSE scores were abnormal in the first four examinations performed while the patient was stuporous, but arousable. Thereafter they showed a progressive improvement, reaching the normal range (score ≥24) ~1.5 days after the patient regained a normal vigilance level. Anterograde memory, attention and writing were the MMSE items which were the last to return to normal levels. A similar pattern of serum endozepine-4 was seen in Case 17. In both patients, endozepine-4 fluctuations occurred over 20–24 h for 2–3 days.

Treatment of endozepine stupor

Intravenous administration of 0.5–2 mg of flumazenil awakened all the patients and normalized the EEG (shifting the frequency peak from the beta to the alpha range) (Fig. 3) in every case. However, the effect was transient, lasting only 10–15 min. Thereafter patients lapsed again into stupor, and the EEG became fast and unreactive. We tried a prophylactic treatment of endozepine stupor in an open trial with oral flumazenil 20 mg/day in five patients (Cases 12, 16, 17, 19 and 20), who gave informed consent to the treatment. In two cases (Cases 17 and 20), side effects (nausea, anxiety) terminated the study after a few days. In the remaining three cases, characterized by a frequency of 1–6 or >6 attacks per year prior to the trial, only one minor attack for each patient was observed during >1 year of treatment.

Discussion

Our findings show that endozepine stupor is non-hereditary and occurs predominantly, but not exclusively, in middle-aged men. Since our patients come from throughout Italy and have different occupations, a common environmental or occupational origin is unlikely. Similarly, there does not appear to be any predisposing physical or mental disturbance. The duration and intensity of stupor episodes vary in different patients and from one episode to the next. Stupor may be preceded or followed by aggressive or depressive behaviour, confusion, amnesia, confabulation, dysartria, ataxia and/or psychomotor slowing. Retro- and anterograde memory loss persists after each episode. Outside of Italy, endozepine stupor has been observed in a 42-year-old Canadian woman with a 20-year history of the disorder associated with high levels of endozepine-4 (Chen et al., 1995). A patient living in South Africa with a positive family history of recurring unconsciousness episodes resolved by flumazenil (Lotz et al., 1993) also had high endozepine-4 levels during stupor (J. D. Rothstein, personal observation), though EEG showed diffuse alpha activity during the episode. Thus, endozepine stupor is not restricted to Italy, but only future studies will determine whether it is ubiquitous or prevails in some populations. Our patients had received extensive examinations in prior hospitalizations, and had been variably diagnosed as self-intoxicated, drunk, psychogenic unresponsive, demented, etc. In some cases, intentional intoxication by relatives had been covertly suggested by the treating physicians, with disastrous consequences on family life. A correct early diagnosis of endozepine stupor is therefore mandatory. In all the nine patients studied with plasma and CSF analysis of endozepine-4, the ligand levels during the stupor episodes were similar to diazepam levels high enough to produce stupor (Mennini and Garattini, 1983; Haefely et al., 1985). When sampled between attacks, plasma and CSF levels of endozepine-4 were normal or undetectable. Although recurring stupor episodes can be provoked by commercially available halogenated benzodiazepines, such as diazepam and its metabolites, HPLC and mass spectrometry (four subjects) never detected synthetic benzodiazepines in serum or CSF samples of our endozepine stupor patients. Thus, an ictal increase in endozepine-4 seems to represent the direct and sole origin of endozepine stupor.

Endozepines are a class of non-benzodiazepine, non-protein molecules, recently found to act as positive allosteric modulators of GABA_A receptors and mimicking the pharmacological activity of exogenous benzodiazepines. In the past, the term endozepine has been associated either with
Fig. 4 Serial endozepine-4 blood levels (closed circles) and MMSE scores (squares) in Case 20. Before stupor, there was no endozepine-4 in plasma. Nine hours from the onset of stupor the endozepine-4 level was markedly high and displayed significant fluctuations during the 3.5 days of stupor. The MMSE scores were altered (score <24) in the first six examinations during stupor, entered the normal range (score ≥24) at the seventh examination 1.5 days after the patient regained consciousness and reached the baseline interictal patient’s performance (determined at day 17) only at the ninth examination, 3.5 days after the end of the stupor.

Endozepine-4, like synthetic benzodiazepines, has a high affinity for the GABA<sub>A</sub> receptor (Rothstein et al., 1992c), and this may explain its powerful pharmacological effect and its ability to induce coma. In particular, benzodiazepine receptor agonists like endozepine-4, have a well described affinity for the hippocampal GABA receptors, and this could also explain the prominent disturbances of memory we observed in the prodromal and recovery phases.

The cause of the raised concentrations of endozepine-4 in endozepine stupor remains to be elucidated. The sudden huge accumulation of this compound must occur, however, outside the brain, otherwise there would be no explanation for such a high plasma concentration detected during the stuporous episodes. This rise could be due to abnormal enzymatic degradation, or to a sharp increase in its precursor supply. Benzodiazepines and their precursors can be produced by selected fungi or bacteria, ubiquitous in the environment and in our food chain (Luckner, 1984; Wildmann et al., 1988; Medina et al., 1989; Unseld et al., 1989), and endozepine-4 or some of its precursors could accidentally enter the patients’ diet. Endozepine-4, together with endozepine-2 is also increased during episodes of hepatic encephalopathy (Olasmaa et al., 1990) in which a partial effect of flumazenil has also been described (Scollo-Lavizzari and Steinmann, 1985). Therefore we investigated liver function, in particular ammonium levels, by liver echography and biopsy studies, but found no obvious liver dysfunction nor peculiar dietary habits in our cases. Besides hepatic encephalopathy, it remains unknown whether endozepines are increased in other human disorders characterized by decreased vigilance and resembling endozepine stupor. The Kleine–Levin syndrome and in particular narcolepsy, are both conditions characterized by recurring episodes of somnolence and may, in selected cases, mimic the recurring stupors observed in our patients. In the Kleine–Levin syndrome no information is available on endozepine levels; however, the episodes persist for 1 or more weeks and somnolence is not normally associated with mental confusion (Critchley, 1962). Moreover, somnolence in the Kleine–Levin syndrome is attended by physiological sleep patterns, with normal sleep rhythms and cycling and slowing on the EEG (Parkes, 1985), even in clinical variants with incomplete forms of the syndrome (Billiard, 1990). Ictal EEG therefore differentiates endozepine stupor from the Kleine–Levin syndrome. Since, however, the exact boundaries of both conditions are still unclear, patients presenting with fast EEG activity and disturbances resembling the Kleine–Levin syndrome should be investigated for endozepine levels. Conversely, endozepine stupor is not associated with the oneiric mental activity typical of the narcoleptic attacks, and endozepine-4 levels are normal in conditions such as narcolepsy and sleep apnea (Montagna et al., 1995).

Clarification of endozepine stupor pathogenesis must await the characterization of the chemical structure of endozepine-
4 and its metabolic pathways in humans. If endozepones are shown to represent endogenous neurotransmitters, analogous to the endogenous opiate, cannabinoid or pyridine systems, endozepine stupor may offer insights into a group of novel metabolic pathways and functional brain disturbances. At the moment, flumazenil treatment to fend off the endozepine stupor episodes, though promising, is still too preliminary and needs rigorous trials.

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