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Developmental and neurochemical effects of early postnatal exposure to flumazenil in female and male mice

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The effect of the benzodiazepine antagonist flumazenil (10 and 20 mg/Kg) on righting reflex, body weight, body temperature, and proteins, cholesterol and phospholipids of the brain was examined in new born male and female mouse pups. In males, the level of brain cholesterol was increased by the low dose but diminished by the high dose. On the contrary, the amount of phospholipids and protein was diminished by the low dose and increased by the high dose. In females, the level of phospholipids was reduced by 10 mg/Kg but was increased by 20 mg/Kg. Flumazenil dose-dependently decreased the level of proteins, although the quantity of brain cholesterol was similar to males Postnatal exposure to flumazenil did not influence righting reflex, body weight and body temperature although prenatal exposure to the drug retarded the formermentioned response. Postnatal flumazenil clearly had a very profound effect on biochemical measures in mice, which could reflect the antagonist's powerful fluidising effects on neuromembranes.

Efectos neuroquímicos y sobre el desarrollo de la exposición postnatal temprana al flumazenil en ratones machos y hembras. Se estudia el efecto del antagonista benzodiacepínico flumazenil (10 y 20 mg/Kg) en el «righting reflex» (incorporación), peso y temperatura corporal, proteínas, colesterol y fosfolípidos cerebrales en crías de ratones de ambos sexos. En los machos, la dosis baja incrementa el nivel de colesterol, disminuyendo los fosfolípidos y las proteínas. Contrariamente, la dosis alta disminuye el nivel de colesterol incrementando fosfolípidos y proteínas. En hembras, la dosis de 10 mg/kg disminuye el nivel de fosfolípidos, mientras que la de 20 mg/kg lo incrementa. El flumazenil disminuye de forma dosis-dependiente el nivel de proteínas, aunque con respecto al colesterol cerebral los resultados fueron similares a los machos. La exposición postnatal al flumazenil no afecta al «righting reflex», el peso corporal o la temperatura. La exposición postnatal tiene un profundo efecto en las medidas bioquímicas de los ratones lo cual podría reflejar una poderosa acción en la permeabilidad de las neuromembranas.

Flumazenil (Ro 15-1788), is a benzodiazepine antagonist with intrinsic dose-dependent properties that affect behaviour. At a high dose (50 mg/kg), it is anticonvulsant (Nutt and Cowen, 1982), but at low doses (10 mg /Kg), it has an anxiogenic action (File and Pellow, 1985). Flumazenil crosses the placental barrier and can easily and quickly pass through the blood-brain barrier (Lister, Greenblatt, Abernethy and File, 1984), thus potentially modifying the developing central nervous system (CNS) in the neonate. Several studies have shown that perinatal exposure to benzodiazepines can affect early development and adult behaviour (Alleva, Laviola, Tirrelli, and Bignami, 1985; Benton, Dalrymple-Alford, Brain and Grimm, 1985; Gai and Grimm, 1982; Brain, Ajarem and Petkov, 1987; Pankaj and Brain, 1991a). Perinatal flumazenil treatment in pregnant rats has produced effects on emotivity-related behaviours of adult offspring (Fernández-Teruel, Driscoll, Escorihuela, Tobeña and Battig, 1993). Administration of this benzodiazepine antagonist to pregnant dams of a selected line of rats bred for poor acquisition of shuttle box performance, improved this task in adult subjects (Ferré, Escorihuela, Tobeña and Fernandez-Teruel, 1996). The authors argued that this effect could be due to a reduction of anxiety or to an improvement of memory in those animals. On the other hand, in a recent study, flumazenil did not produce a significant effect on rat pup ultrasonic vocalisation (Olivier, Molewijt, Van-Oorschot, Van-der-Heyden, Ronken and Mos,1998).

Grimm, McAllister, Brain and Benton (1984) assessed the utility of the posture analysis in mice to assess how pre- and early postnatal exposure to benzodiazepine influences later social interaction. It has been observed that pre and early post-natal exposure to psychoactive drugs influences later social interactions. Prenatally exposing males to diazepam changes their social behaviour (Brain, Ajarem and Petkov, 1986). On the other hand, prenatal administration of different benzodiazepine agonists and antagonists impairs the righting reflex as well as subsequently modifying social behaviour (Pankaj and Brain 1991a, b). During postnatal development, dramatic changes occur in the brain and its constituents. These disturbances may produce changes which are accompanied by behavioural abnormalities. Changes in cho-

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lesterol, phospholipid and protein content can have dramatic effects on the physico-chemical properties of membranes (in particular causing membrane fluidity to vary substantially), This, in turn, would alter the biological function of the membrane (notably having a powerful effect on neurophysiological sensitivity). Furthermore, exposure of biological membranes to agents such as the benzodiazepines (commonly prescribed to treat anxiety in female patients) are capable of modifying lipid fluidity perhaps precipitating homeoviscous adaptation of the membrane which could then persist even once the benzodiazepine has been eliminated from the body (Kurishingal, Brain and Restall, 1992). During neuronal development and myelination, significant changes may occur in the membrane properties. For example, a myelinated membrane is rich in cholesterol and consequently highly ordered. Such a system may be relatively resistant to the effects of agents such as the benzodiazepines compared to a membrane that has not been myelinated. There may, therefore, be very different consequences of exposing the organism to benzodiazepines before or after the myelinisation process. The brain varies in its susceptibility to teratogenic substances, depending on the biochemical changes that are occurring. The aim of this work was to assess whether the benzodiazepine antagonist flumazenil (with its intrinsic properties) produced lasting effects in mouse neonates (it has been argued that CNS development in the newborn mouse is comparable to that of the human in the third trimester of pregnancy) simulating the «floppy infant syndrome»-like characteristics induced by certain benzodiazepines. This syndrome in human neonates is characterised by hypothermia, hyperbilirubinaemia, hypotonia, asphyxia and breathing disorders. This has been linked to diazepam treatment in pregnancy and has been modelled in rodent studies (Grimm et al., 1984; Pankaj and Brain, 1991a). The question we are seeking to answer is whether treatment with the antagonist flumazenil, that is used to reverse benzodiazepine effects (following their use in anaesthesia or after over-dosing), has some of the behavioural and physiological consequences as benzodiazepine administration (Pankaj and Brain 1991b). An attempt is also made to establish a mechanism for these persistent behavioural teratological effects. This study examined the effects of two doses (10 and 20 mg/Kg) of flumazenil on several behavioural measures, righting reflex, body weight and body temperature, and several biochemical measures of brain constituents (proteins, cholesterol and phospholipids), in male and female mouse pups.

Materials and Methods

One hundred and ninety two CD1 strain mouse pups were used in this study. Twenty one males and 21 females were used for behavioural measures, and 75 males and 75 females for the biochemical determinations. Animals were obtained from the animal facility of the University of Wales Swansea. The subjects were allocated to opaque polypropylene cages measuring 35 x 15 x 13 cm (North Kent Plastics, U.K.), kept under reversed lighting conditions (white lights on, from 11:30 to 10:30 hrs G.M.T.) and at a controlled temperature (18-21°C). Food (Pilsbury's Breeding Diet, Birmingham, U.K.) and water were provided *ad libitum.*

Procedures involving mice and their care were conducted in conformity with national, regional and local laws and regulations, which are in accordance with the European Communities Council Directives (86/609/EEC, 24 November 1986).

Drug Treatments

All subjects were given intraperitoneal injections with control vehicle (one drop of Tween 80 in 0.9% sodium chloride) or 10 or 20 mg/kg of flumazenil (Roche Products Ltd, Welwyn Garden City, Herts U.K.) in the vehicle. Injections were given on the first day of postnatal life in a single administration, and pups were then quickly returned to their litters. The drug solutions were stored in the dark and were injected at the same temperature as the experimental subjects. The injected volumes were standardised at 0.1 ml per 20 g body weight. All litters were adjusted so they consisted of 3 males and 3 females, with one animal of each sex in each drug treatment group (subjects were marked). The doses were chosen on the basis of a previous study (Pankaj and Brain 1991b) in which the effects of prenatal exposure to benzodiazepine-related drugs on early development and adult social behaviour was studied in mice. In addition, these doses are used by other authors in postnatal studies on rodents (File and Lister, 1983; File and Pelow 1985; Hoffman and Britton, 1983; Taylor, Little, Nutt and Sellars, 1985).

Developmental measures

Males and females were assessed as separate categories. The developmental measures studied on the first 5 days of postnatal life were:

Righting Reflex: The righting response consists of the time taken for a pup placed on its back to turn over and place all four paws on the substrate. An upper limit of 2 min was set for this test. This time required by an animal to right itself was recorded using a stopwatch (Holder, Stanhope-Seta). This test was conducted between 11:00 and 13:00 hours GMT. The behavioural measure was made after the treatment on day 1.

Body Weight: Since weight is a useful indicator of physical development, pups were weighed each day using a digital balance (Model PE 3600 Metler, Switzerland).

Body Temperature and Biochemical measures

Five litters were used for these measurements on each of the first 5 days of postnatal life. Males and females were assessed as separate categories.

Body temperature: The subjects were decapitated as soon as they were removed from the rest of the litter. The core temperature was obtained immediately post-decapitation by introducing the probe of the digital thermometer into the animal's rectum. It is possible to obtain an accurate measure of this index, reflecting the poikilothermic nature of the pups, until they are 6 days of age (Okon 1970).

Proteins: The procedure used to assay proteins was a modification of the method of Bradford (1976). The whole brain was homogenised in 1 ml of buffer (200 mM Tris-HCl pH 7.4). Half of this sample was diluted in 450 ml of distilled water. 100 ml of this brain sample was further diluted 1:10 before being mixed with 5 ml of Coomassie blue (50 mg Coomassie brilliant blue + 25 ml 95% ethanol +50 ml of orthophosphoric acid + 425 ml distilled water) left for 5 minutes before measuring the absorption at 595 nm in a spectrophotometer (Cecil Instruments 5501). The protein content of the sample was estimated from a standard curve.

Cholesterol: There are several methods to determine cholesterol but many are prone to interference (Tonks 1967). Sigma Diag-

nostics Cholesterol Reagent (Sigma Chemical Company, Poole, Dorset, U.K.) measures cholesterol enzymatically and is a modification of the method of Allain, Poon, Chan, Richmond and Fu (1974). For the cholesterol procedure, 10 µl of brain with a protein concentration of 2 mg/ml was used. This sample was mixed with 1 ml of cholesterol reagent and incubated for 5 minutes at 37ºC. Subsequently, the absorption was determined at 500 nm and the cholesterol content of the sample estimated using a standard curve.

Phospholipids: To obtain phospholipid titres, 2.5 ml of 7.5% ammonium molybdate and 0.2 ml of 11% Tween 20 were added on the day of use to 10 ml of colour reagent, which was prepared by dissolving 0.44 g of Malachite Green in dilute sulphuric acid (60 ml sulphuric acid mixed with 300 ml distilled water) which had been chilled on ice (once prepared, the colour reagent is stable for over 1 year). 0.2 ml of this solution was mixed with 0.2 ml of sample and left for 10 minutes before the absorption measured at 630 nm. The phospholipid content of the sample was estimated using a standard curve.

Statistical analysis

Firstly, the data for the biochemical measures were analysed using a mixed analysis of variance, for each measure, with two «between» factors (treatment and gender). Secondly, developmental and biochemical measures were analysed using a mixed analysis of variance, with two «between» (treatment and gender) and one «within» (days) factors. Where appropriate, post- hoc Newman-Keuls tests were carried out to evaluate differences between pairs of results.

Results

A. Data of the Biochemical measures taken together

The mean of biochemical measures for male and female pups on the first 5 days are shown in Figures 1, 2 and 3.

Only the interaction between Sex x Treatment [F (2,144) = 4.952; p< 0.0083] was significant with respect to brain proteins. Simple main effects show that Sex was significantly different in the saline and flumazenil 10 mg/kg groups (p< 0.032 and p<0.017, respectively). The effect of treatment was only evident in females (p< 0.025). An ANOVA of the female treatment groups was performed [F (2,72) = 3.340; p< 0.0410], but Newman-Keuls analysis did not show differences between groups.

Sex [F (1,144) = 7.686; p< 0.0063] and Treatment [F (2,144) = 6.149; p< 0.0027] (but not their interaction), had significant effects on brain cholesterol. Females presented lower levels than males (p< 0.01) and the highest dose of flumazenil significantly decreased brain cholesterol with respect to saline (p< 0.05) or the lower dose (p< 0.01).

Only Treatment had a significant impact on brain phospholipids [F (1,120) =1014.97; p< 0.0001], the lower dose of flumaze-

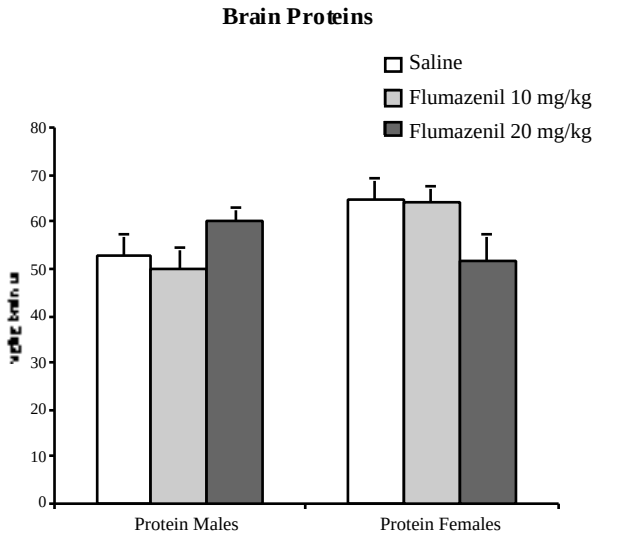

Figure 1. *Means (±S.E.) of brain proteins of male and female pups of the first five days of postnatal life after a single treatment on the day of birth with control or one of two doses of flumazenil*

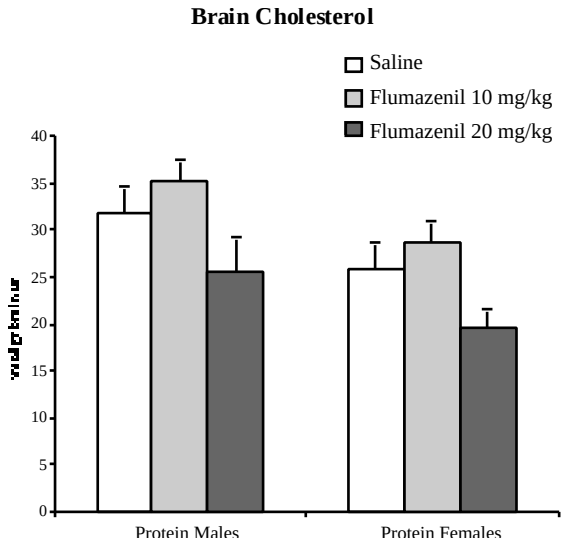

Figure 2. *Means (±S.E.) of brain cholesterol of male and female pups of the first five days of postnatal life after a single treatment on the day of birth with control or one of two doses of flumazenil*

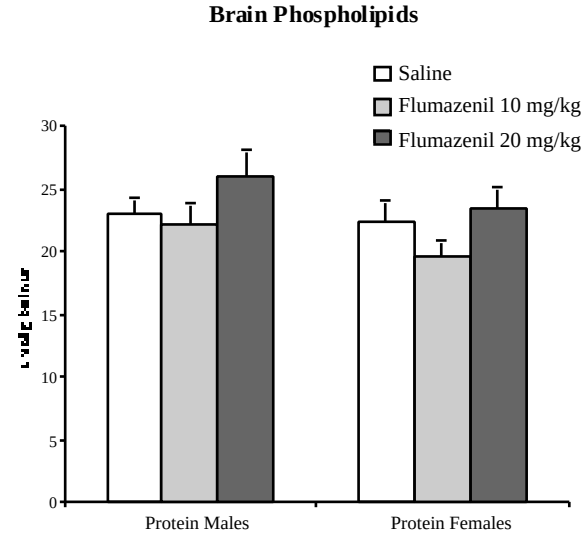

Figure 3. *Means (±S.E.) of brain phospholipids of male and female pups of the first five days of postnatal life after a single treatment on the day of birth with control or one of two doses of flumazenil*

nil showing a significant decrease with respect to the levels obtained with the higher dose.

B. Data of developmental, core temperature and biochemical measures on each of the first 5 days

The mean developmental and core temperature plus biochemical measures for male pups on each of the first 5 days are shown in tables 1 and 2, respectively. Tables 3 and 4 show comparable data for female pups

The comparisons between developmental and biochemical measures in male and female pups were subsequently made.

Neither Sex nor its Interaction with Age and Treatment had significant effect on body weight or core temperature. ANOVA revealed that only Sex presented a significant effect on righting reflex [F (1,36) =5.925; p< 0.02], with ma-

Table 1

Means (±S.E.) of developmental measures (righting reflexes, body weights and body temperatures) of male pups over the first 5 or 6 days of postnatal life after a single treatment on the day of birth with control or one of two doses of flumazenil

	Righting Reflex (sec)			Body Weight (g)			Body Temp. (ºC)		
Postnatal days	Saline	Flumazenil 10 mg/kg	Flumazenil 20 mg/kg	Saline	Flumazenil 10 mg/kg	Flumazenil 20 mg/kg	Saline	Flumazenil 10 mg/kg	Flumazenil 20 mg/kg
1	19.4±6.74	21.4±5.69	22.2±6.1	1.7±0.069	1.7±0.081	1.7±0.059	31.1±0.75	30±0.95	30.2±0.5
2	10.8±2.19	16.4±3.86	11.2±2.22	2±0.045	2±0.14	2±0.1	28.9±0.12	31.1±0.94	24.7±0.68
3	10.7±4.08	12.7±3.81	13.7±2.88	2.4±0.094	2.5±0.21	2.4±0.15	32.1±0.4	32.3±0.56	32±0.26
4	5.1±1.75	5.2±1.64	8.7±3.42	2.9±0.13	3±0.27	2.9±0.2	33.3±0.37	32.5±0.21	32.2±0.2
5	3.7±0.8	3.8±1.46	13.1±4.73	3.6±0.14	3.6±0.29	3.5±0.22	33±0.29	32.5±0.19	31.9±0.32
6	5.5±3.54	5.7±4.04	5±1.53	4.3±0.15	4.3±0.36	4.2±0.25			

Table 2

Means (±S.E.) of biochemical changes (brain proteins, cholesterol and phospholipids) of male pups over the first 5 or 6 days of postnatal life after a single treatment on the day of birth with control or one of two doses of flumazenil

	Brain Proteins (mg/g brain ut)			Brain Cholesterol (nmol/g brain ut)			Brain Phospholipids (mmol/g brain ut)		
Postnatal days	Saline	Flumazenil 10 mg/kg	Flumazenil 20 mg/kg	Saline	Flumazenil 10 mg/kg	Flumazenil 20 mg/kg	Saline	Flumazenil 10 mg/kg	Flumazenil 20 mg/kg
1	70.2±1.78	87±0.35(*)	82±0.75(*)	21±0.7	7±0.35(*)	19±0.7	24.8±0.22	20.4±0.45(*)	23±0.75
2	46±0.35	52±0.75(*)	29±0.35(*)	14±0.35	36±0.35(*)	18±0.35(*)	26.2±0.75	26.2±0.4(*)	29±0.35
3	57±0.75	61.4±0.45(*)	77±0.93(*)	55±0.6	39±0.35(*)	5±0.7(*)	13.6±1	13.6±0.45(*)	18±0.35
4	54±0.75	41±0.35(*)	45±0.75(*)	24±0.35	28±0.7(*)	18±0.8(*)	11.8±0.35	11.8±0.41	14±0.35(*)
5	98±0.75	77±0.70(*)	25±0.5(*)	16±0.7	35±0.6(*)	39±0.35(*)	26±0.5	26±0.35(*)	33±0.93(*)

Table 3

Means (±S.E.) of developmental measures (righting reflexes, body weights and body temperatures) of female pups over the first 5 or 6 days of postnatal life after a single treatment on the day of birth with control or one of two doses of flumazenil

	Righting Reflex (sec)			Body Weight (g)			Body Temp. (ºC)		
Postnatal days	Saline	Flumazenil 10 mg/kg	Flumazenil 20 mg/kg	Saline	Flumazenil 10 mg/kg	Flumazenil 20 mg/kg	Saline	Flumazenil 10 mg/kg	Flumazenil 20 mg/kg
1	29±16.6	11.7±2.72	43.4±21.55	1.6±0.049	1.44±0.73	1.64±0.05	28.9±1.05	28.5±1.1	28.6±1
2	13.4±3.93	128±6.7	12.5±3.4	1.88±0.1	1.7±0.12	1.98±0.1	30.3±0.65	29.6±0.81	29.5±0.57
3	8.7±2.62	31.4±16.6	11.8±3.21	2.32±0.16	2±0.18	2.4±0.15	30.7±0.85	31.4±0.33	30.5±0.55
4	16.1±1.65	18.5±7.74	25.1±17.41	2.85±0.2	2.52±0.23	2.94±0.21	31.6±0.27	31.9±0.07	31.8±0.13
5	12±2.8	14.2±6.64	11.8±7.17	3.38±0.23	3±0.25	3.5±0.22	32.1±0.25	31.9±0.38	31.4±0.35
6	5.5±1.9	5.4±2.16	9±3.87	4.15±0.27	3.77±0.3	4.22±0.27			

Table 4

Means (±S.E.) of biochemical changes (brain proteins, cholesterol and phospholipids) of female pups over the first 5 or 6 days of postnatal life after a single treatment on the day of birth with control or one of two doses of flumazenil

	Brain Proteins (mg/g brain ut)			Brain Cholesterol (nmol/g brain ut)			Brain Phospholipids (mmol/g brain ut)		
Postnatal days	Saline	Flumazenil 10 mg/kg	Flumazenil 20 mg/kg	Saline	Flumazenil 10 mg/kg	Flumazenil 20 mg/kg	Saline	Flumazenil 10 mg/kg	Flumazenil 20 mg/kg
1	77.2±0.41	47±0.7(*)	67±0.75(*)	54±0.7	39±0.35(*)	57±0.7(*)	23.4±0.57	18.8±0.41(*)	16±0.75(*)
2	69±0.7	60±1.5(*)	62±0.61(*)	35±0.7	26±0.4(*)	23±0.7(*)	23±0.75	21±0.5	32±0.35(*)
3	31±0.35	64.4±0.26(*)	37±0.5(*)	18±0.6	52±0.35(*)	32±0.6(*)	20±1	9±0.35(*)	22±0.7
4	59.6±0.75	15±0.35(*)	75±0.35(*)	25±0.6	37±0.7(*)	7±0.7(*)	18±0.79	30±1.25(*)	41.6±0.57(*)
5	30±0.35	67±0.75(*)	59±0.35(*)	28±0.35	24±0.6	10±0.7(*)	33±0.35	31.6±0.57	22±0.93(*)

les having faster reflexes than females (Newman-Keuls, $p< 0.01$).

Sex [$F(1,120) = 519.276$; $p< 0.0001$] and the interactions between Age x Sex [$F(4,120) = 1084.636$; $p< 0.0001$] and Sex x Treatment [$F(2,120) = 845.326$; $p< 0.0001$] were all significant with respect to brain proteins. Furthermore, females had higher levels than males after saline or 10 mg/kg of flumazenil but the 20 mg/kg dose produced a greater decrease in females ($p< 0.01$). Sex [$F(1,120) = 1014.97$; $p< 0.0001$] and the Interaction between Age and Sex [$F(4,120) = 1338.61$; $p< 0.0001$] were both significant influences on brain cholesterol. Males had higher levels than females each day ($p< 0.01$) except on the fourth postnatal day. Treatment influenced males and females in terms of their level of brain cholesterol differently (ps< 0.01). Sex [$F(1,120) = 114.499$; $p< 0.0001$] and the interactions between Age and Sex [$F(4,120) = 348.708$; $p< 0.0001$] and Sex and Treatment [$F(2,120) = 11.282$; $p< 0.0001$] all had significant effects on brain phospholipids. Males generally had higher levels than females ($p< 0.01$). In addition, males had higher levels of brain phospholipids with both Treatment doses ($p< 0.01$).

Analysis of variance for males revealed that Treatment did not influence body weight, core temperature or righting reflex. A clear age-related effect on these measures was observed.

Age [$F(4,60) = 638.70$; $p< 0.0001$], Treatment [$F(2,60) = 322.14$; $p< 0.0001$] and their Interaction [$F(8,60) = 1266.56$; $p< 0.0001$] all had significant effects on brain protein. Except for the first and second days of postnatal life, brain proteins changed significantly each day (ps< 0.01). The low dose of flumazenil decreased whereas the higher one increased their level (ps< 0.01). It was also found that Age [$F(4,60) = 1228.11$; $p< 0.0001$], Treatment [$F(2,60) = 391.21$; $p< 0.0001$] and Interaction [$F(8,60) = 481.36$; $p< 0.0001$] were all significant factors with respect to brain cholesterol. Overall, it decreased significantly each day (ps< 0.01). The low dose of flumazenil increased the level (ps< 0.01) whereas the higher dose decreased it. Age [$F(4,60) = 238.96$; $p< 0.0001$] Treatment [$F(2,60) = 69.41$; $p< 0.0001$] and Interaction [$F(8,60) = 149.55$; $p< 0.0001$] were all significant for brain phospholipids. With the exceptions of the fourth and the fifth days, they were significantly different on each day ($p< 0.01$). The low dose of flumazenil decreased whereas the high dose increased the level of phospholipids (ps< 0.01).

Analysis of variance for females revealed that Treatment did not influence body weight, core temperature or righting reflex. A clear age-related effect on these measures was observed.

Age [$F(4,60) = 1444.30$; $p< 0.0001$], Treatment [$F(2,60) = 554.74$; $p< 0.0001$] and their Interaction [$F(8,60) = 765.30$; $p< 0.0001$] all had significant effects on brain protein. It was significantly higher the first day than subsequently (ps< 0.01). Flumazenil dose-dependently decreased the level of proteins and progressively altered them over days. Age [$F(4,60) = 493.69$; $p< 0.0001$] Treatment [$F(2,60) = 393.09$; $p< 0.0001$] and their Interaction [$F(8,60) = 815.11$; $p< 0.0001$] were all significant sources of variance in brain cholesterol, which was different every day (ps< 0.01). The low dose of flumazenil increased whereas the high decreased the level of cholesterol. Age [$F(4,60) = 661.13$; $p< 0.0001$] Treatment [$F 2,60) = 79.90$; $p< 0.0001$] and their Interaction [$F(8,60) = 10.07$; $p< 0.0001$] all significantly influenced brain phospholipids. The low dose of flumazenil decreased whereas the high dose increased their level (ps< 0.01).

Discussion

Treating pregnant mice with flumazenil (10 or 20 mg/kg) retards the righting reflexes and increases the ultrasonic distress calling of their offspring (Brain, Kurishingal and Restall, 1995). Flumazenil administered prenatally, also increases the amount of cholesterol and reduces the phospholipids in the brains of offspring (Brain *et al*, 1995). Although in the present study clear age-related effects on the righting reflex, body weight, and body temperatures were apparent, postnatal exposure to flumazenil did not influence these measures. These differences (in relation to the righting reflex) from prenatal exposure (where the substance retarded the response) may be due to the timing of exposure or the fact that benzodiazepines often persist for a relatively long time in the foetus. The effective doses could also be different. Clearly a wide range of subtle behaviours may well have been changed by postnatal exposure to flumazenil (Grimm *et al*, 1984).

Treatment with postnatal flumazenil clearly had a very profound effect on brain proteins, cholesterol and phospholipids in male and female mice, which could well reflected the antagonist's powerful fluidising effects on neuromembranes. It is notable that Kurishingal (1994) showed that flumazenil given to pregnant female mice increased cholesterol but decreased phospholipid and protein content in the brains of their offspring. When the biochemical measures were analysed in their totality, the present results differed from those obtained after prenatal treatment. There was a decrease in cholesterol after the higher dose of flumazenil, conversely to the increase observed when it was given prenatally. No significant changes were observed in phospholipids and proteins, which only decreased in females after the higher flumazenil dose administration. Analysis of the measures from the present study, on each of the first 5 days also differs from prenatal administration. Giving the drug directly to the neonate decreased cholesterol. Phospholipids and proteins were reduced by the lower but augmented by the higher dose of flumazenil. These differences may well be associated with the timing of the exposure and/or the effective doses present in the brain. These effects may be quantitatively different in males and females as the former had higher levels of cholesterol and phospholipids and the latter higher levels of protein. It is difficult, however, to rule out the possibility that the difference between the prenatal and postnatal effects of this drug is a consequence of dose rather than timing. We cannot establish with accuracy how much of the drug reaches the foetal brain following treatment of the mother. The postnatal application may be the more clinically relevant and it is easier to ensure that the doses given are accurate in these animals.

It is well established that subtle influences (such as the very act of injection) can have powerful effects on developing organisms. The mouse is born in a relatively undeveloped state being initially blind and capable of simple movements (towards a teat or towards the centre of a mass of siblings). They are basically poikilothermic and only capable of simple righting reflect and ultrasonic distress calling (Nastiti, Benton and Brain, 1991) which encourages the mother to retrieve them for protection, thermoregulation and feeding. The studies carried out here, contrast animals treated with placebo or one of two doses of flumazenil. The biochemical and behavioural changes seen in the placebo-treated animals are broadly similar to those seen in untreated controls in this strain of mouse. It is argued that subtle influences by the drug could not only produce long-term changes in the functioning of the CNS but

could (by changing the timing of key events in the lives of the subjects) alter the quality of parental care and the timing and influences of so-called sensitive periods. An understanding of such complex but relatively pervasive effects seems of great importance to our understanding of physician-induced disease caused by the use of benzodiazepines.

In terms of relating the rodent model to the clinical condition, it is pertinent to note that the rodent neonate's brain is less mature at parturition than that of its human counterpart. It has been suggested by exponents of behavioural teratology that in the first few days of postnatal life, the brain of a rat or a mouse is roughly equivalent to a foetus in the third trimester of pregnancy. This is the period in which benzodiazepines are thought to exert their influence on the «floppy infant syndrome». Consequently, the postnatal effects observed in the present study may be clinically-relevant, raising several questions about the possibility that flumazenil can create long-lasting perturbations in the development of the brain when used at this sensitive period.

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