British Journal of Pharmacology (2001) 134, 1393–1402

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The interaction between ethanol and pregnanolone at induction of anaesthesia investigated with a threshold method in male rats

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1 An anaesthesia threshold was used to investigate the pharmacodynamic and pharmacokinetic interactions between ethanol and pregnanolone in male rats.

2 The criterion to determine threshold doses of pregnanolone was the first burst suppression of 1 s in the EEG. Ethanol (0.5, 1.0, 1.5 and 2.0 g kg^{-1}) was injected i.p. 15 min before pregnanolone infusion. Trunk blood, serum, cortex, cerebellum, hippocampus, striatum, brain stem, fat and muscle tissues obtained at criterion were used to determine ethanol (blood) and pregnanolone.

3 Ethanol reduced threshold doses in a dose dependent linear manner. A similar reduction of pregnanolone tissue concentrations was only found in brain stem and striatum. Deviations consisted of larger decreases in serum, cerebellum and hippocampus after 0.5 g kg^{-1} ethanol and in cerebellum, cortex and hippocampus after 2.0 g kg^{-1} of ethanol. Positive correlations between dose and concentration of pregnanolone was recorded in brain stem, hippocampus, cerebellum and cortex. A kinetic component influenced the concentration in cortex. There was a correlation between dose and serum concentration of pregnanolone only after ethanol. In the muscle 0.5 g kg^{-1} ethanol had no influence on pregnanolone concentration.

4 The linear, additive pharmacodynamic interaction could involve the GABA ionophore. A pharmacokinetic interaction was found in cortex. The retained high uptake of pregnanolone in muscle (after 0.5 g kg^{-1}) corresponded to losses in other tissues (including serum). The reduced uptake of pregnanolone in cerebellum, cortex and hippocampus (after 2.0 g kg^{-1}) was not due to a corresponding change in serum concentration. It was probably due to a reduced blood flow.
British Journal of Pharmacology (2001) **134,** 1393–1402

Keywords: Ethanol; pregnanolone; anaesthesia threshold; pharmacodynamic interaction; additive interaction; pharmacokinetic interaction; kinetic dose dependence; analysis brain parts

Abbreviations: b, regression coefficient; Q, quotient between brain and serum concentrations of pregnanolone; r, correlation coefficient; SPRD, Sprague Dawley rats (outbred Mol:SPRD Han); SS, a burst suppression in the EEG, which is 1 s or longer

Introduction

Pregnanolone (3α-hydroxy-5α-pregnan-20-one) and allopregnanolone (3α-hydroxy-5α-pregnan-20-one) are endogenous progesterone metabolites and proven to be potent against modulators of $GABA_A$-receptors (Majewska *et al*., 1986; Wang *et al*., 2001). Pregnane steroids are increased in brain at stress (Purdy *et al*., 1991; Barbaccia *et al*., 1996; 1997), as well as during naturally occurring hormone fluctuations such as the menstrual cycle (Finn & Gee 1993; Wang *et al*., 1996) and pregnancy (Löfgren & Bäckström, 1990; Concas *et al*., 1998). A large number of reports demonstrate that the anxiolytic, hypnotic and anaesthetic effects attributed to pregnanolone are mediated by enhancing Cl^-conductance through $GABA_A$-receptors in brain (Wang *et al*., 2001).

The behavioural effects of acute ethanol administration are remarkably similar to the effects of benzodiazepines, barbiturates and neurosteroids, all modulators of $GABA_A$-receptors in the brain. Ethanol is anxiolytic, sedative, anticonvulsant and motor incoordinating (Frye *et al*., 1981; Majchrowicz, 1975). It impairs cognitive processing (Givens & McMahon, 1997; Matthews *et al*., 1995), and acts as an anaesthetic and respiratory depressant at high concentrations (Koch-Weser *et al*., 1976). A number of primary effects of ethanol on the brain have been recorded (Deitrich *et al*., 1989; Eckardt *et al*., 1998). Among these $GABA_A$-receptor modulators this primary inhibitory neurotransmitter system has been identified as one possible key site for the behavioural effects of ethanol. A number of the behavioural effects of ethanol are enhanced by $GABA_A$-receptor agonists and attenuated by antagonists or inverse agonists (Ho & Yu, 1991; Morrow *et al*., 1996). However, it is not clear yet whether ethanol acts directly or indirectly on $GABA_A$-receptors. Understanding the sites of ethanol action that mediate its acute and chronic neural and behavioural effects is critical to finding a treatment for alcoholism.

There exist today a number of indications of interaction between ethanol and neurosteroid action in the central nervous system. Acute ethanol administration (2 g kg^{-1}) to male and oestrus female rats increased the levels of allopregnanolone in plasma and cortex (Barbaccia *et al*., 1999). Such an increase in brain neurosteroid levels is sufficient to enhance GABA-mediated neurotransmission in the brain (Morrow *et al*., 1987; 1990) and produces anxiolytic

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and anticonvulsant effects (Crawley *et al*., 1986; Devaud *et al*., 1996). Thus it was suggested that brain levels of allopregnanolone and pregnanolone produced in response to systemic ethanol administration could contribute to several of the ethanol effects associated with $GABA_A$-receptor modulation. Furthermore, the anticonvulsant and inhibitory effects of ethanol on spontaneous neural activity were completely prevented by finasteride, an inhibitor of steroid synthesis (Morrow *et al*., 1999; VanDoren *et al*., 2000). Allopregnanolone influenced the reinforcing properties of ethanol in an operant behaviour paradigm (Janak *et al*., 1998). Finally, both rats and monkeys responded in a discriminative stimulus task in a manner which suggested that allopregnanolone and low ethanol doses may share intrinsic properties that are GABAergic in nature and do not involve inhibition of NMDA receptors (Grant *et al*., 1996; Bowen *et al*., 1999; Morrow *et al*., 1999).

A few earlier studies revealed that neurosteroids enhanced anaesthetic effects of ethanol. Melchior & Allen (1992) reported that pregnanolone (50 mg kg^{-1}) administered with ethanol (3.5 g kg^{-1}) significantly increased anaesthesia time in male mice. Anaesthesia time in this study was defined as the time between loss of righting reflex and the time at which the mouse was again able to right itself three times in 30 s. Duration of loss of righting reflex was monitored following a combined injection of pregnanolone and ethanol. In a replication of the same experiments, trunk blood sample were obtained at the time righting reflex was regained in mice. The authors found that the blood ethanol level of mice injected with 50 mg kg^{-1} pregnanolone was much lower than those of mice given ethanol alone. Melchior & Ritzman (1992) also reported that dehydroepiandrosterone (DHEA), but not dehydroepiandrosterone-sulphate caused a dose-dependent increase in anaesthesia time induced by ethanol treatment (3.5 g kg^{-1}) in male mice. In the present study, we have investigated the interaction between ethanol and pregnanolone in male SPRD rats in more detail. In this investigation the effect of different doses of ethanol were tested on the threshold doses of pregnanolone needed to induce a burst suppression of 1 s or more in the EEG. This threshold technique has been used to obtain detailed information on the pharmacological properties of a number of anaesthetics (Wahlström, 1966; Korkmaz & Wahlström, 1997) including different steroids (Norberg *et al*., 1987; Zhu *et al*., 2001). In the present study clear indications of both pharmacodynamic and pharmacokinetic interactions were obtained.

Methods

Animals

Thirty-four male Sprague-Dawley rats (MOL: SPRD Han M&B A/S, Ry, Denmark) were used in the present study. The rats, weighing 324 ± 10 g, approximately 62 days old, were housed three in each cage in a room with a constant temperature of 22°C and artificial light (light on 1900 to 0700 h). They had access to water and standard food *ad libitum*. They had been accustomed to the new environment for a week before any experiments. The study had been approved by the Regional Ethics Committee for Animal Experiment in Umeå (Umeå djuförsöksnämnd).

Drugs

Pregnanolone (Sigma Chemical Co. St. Louis, MO, U.S.A.) was dissolved in 20% hydroxypropyl-β-cyclodextrin (cyclodextrine) (Sigma Chemical Co. St. Louis, MO. U.S.A.) at a concentration of 4.0 mg ml^{-1}. The preparation was placed in the Bransonic 210 ultrasonic bath for approximately 15 h and agitated occasionally. All steroids were dissolved in cyclodextrin by visual inspection. Ethanol (AB Svensk Sprit, Sweden) was diluted by saline before systemic administration.

Experimental procedure

Saline as vehicle ($n=8$) or ethanol solution in doses of 0.5 g kg^{-1} ($n=7$), 1.0 g kg^{-1} ($n=7$), 1.5 g kg^{-1} ($n=6$, one rat excluded due to unsuccessful infusion) and 2.0 g kg^{-1} ($n=6$), were given i.p. at 15 min before the i.v. infusion of pregnanolone solution at a dose rate of 4.0 mg kg^{-1} min^{-1}. The continuous infusion of pregnanolone was terminated when the anaesthetic criterion of a burst suppression of 1 s or more (the 'silent second', abbreviated SS) was obtained and the rats were immediately killed by decapitation.

Probably due to the fact that ethanol has a large number of effects in the brain (Deitrich *et al*., 1989; Eckardt *et al*., 1998) and complex effects on brain circulation (Altura & Altura, 1996) it cannot be used to induce the SS without reaching a concentration at which the animals die (Wahlström, unpublished). For this reason administration of fixed doses of ethanol was in the present experiments combined with induction of anaesthesia with pregnanolone.

The isobologram is a strong tool to record the pattern of interactions between different drugs (Berenbaum, 1989; Gessner, 1995) and it was used in the present experiments. In a standard isobologram the curves recorded in Figure 1c,d should have reached the abscissa with an intercept corresponding to the dose of ethanol needed to induce the SS, but in our test situation with fixed doses of ethanol the isobologram must be left with an open end.

The time interval between the injection of ethanol and the start of the induction of anaesthesia was 15 min. This rather short interval was chosen to avoid induction of acute tolerance to ethanol (Mellanby, 1919; LeBlanc *et al*., 1975; Wahlström & Widerlöv, 1971), which could have changed the pharmacodynamic situation. Including acute tolerance in the experimental system by increasing the time between ethanol injection and induction of anaesthesia will introduce a new source of variability which might also change the pattern of the interaction.

EEG threshold methods

The anaesthetic effects of pregnanolone were determined with an intravenous EEG-threshold method (Korkmaz & Wahlström, 1997). Pregnanolone solution was infused i.v. *via* a tail vein at a constant infusion rate (4 mg kg^{-1} min^{-1}) and the EEG was recorded continuously from subcutaneous stainless steel electrodes. The infusion was stopped at the first SS and the rats were immediately killed. The appearance of SS occurs at a deeper level of anaesthesia than loss of righting

reflex. The time to reach the SS was recorded and the dose of pregnanolone needed to induce the SS was calculated. This dose is the threshold dose. Dose-rate curves generated earlier (Norberg *et al*., 1987; Zhu *et al*., 2001) by determinations with infusions at different dose rates showed an optimal dose rate of 4.0 mg kg^{-1} min^{-1}, which is associated with the lowest threshold dose of pregnanolone needed to induce the SS. This optimal dose rate was used in the present experiments.

Tissue sample preparation

The rats were killed by decapitation after the first SS was recorded. Trunk blood was collected and the brain was dissected immediately into the following parts: cerebellum, cortex, hippocampus, brain stem and striatum largely according to Glowinski & Iversen (1966). The blood vessels on the surface of the brain were carefully removed before dissecting the brain. Fat tissue from the retroperitoneal abdominal area and tissue from the ileopsoas muscle were removed from each rat. After weighing, the tissue was frozen at $-70°C$ until analysis. The serum was extracted using diethyl ether and tissue samples were extracted with 99.5% ethanol for 7 days at $+4°C$. The recovery of steroids by this procedure was shown previously to be 100% (Bixo *et al*., 1984).

Celite chromatography and hormone assay

Pregnanolone in tissue and serum extracts were purified with celite chromatography, as described earlier (Bäckström *et al*., 1986; Corpéchot *et al*., 1993; Sundström *et al*., 1998). Recovery of pregnanolone was 85%. The concentration of pregnanolone in brain parts, serum, fat and muscle tissues were measured by radioimmunoassay (RIA). Pregnanolone antiserums were raised against 3α-hydroxy-20-oxo-5β-pregnan-11α-yl carboxymethyl ether coupled to bovine serum albumin. The antiserum was kindly provided by Dr Robert Purdy, VAMC La Jolla, San Diego, CA, U.S.A. The specificity and cross-reaction of this antiserum has been verified earlier (Purdy *et al*., 1991; Sundström *et al*., 1998). The sensitivity of the assays was 25 pg, with an intra-assay coefficient of variation of 7% and inter-assay coefficient of variation of 8%.

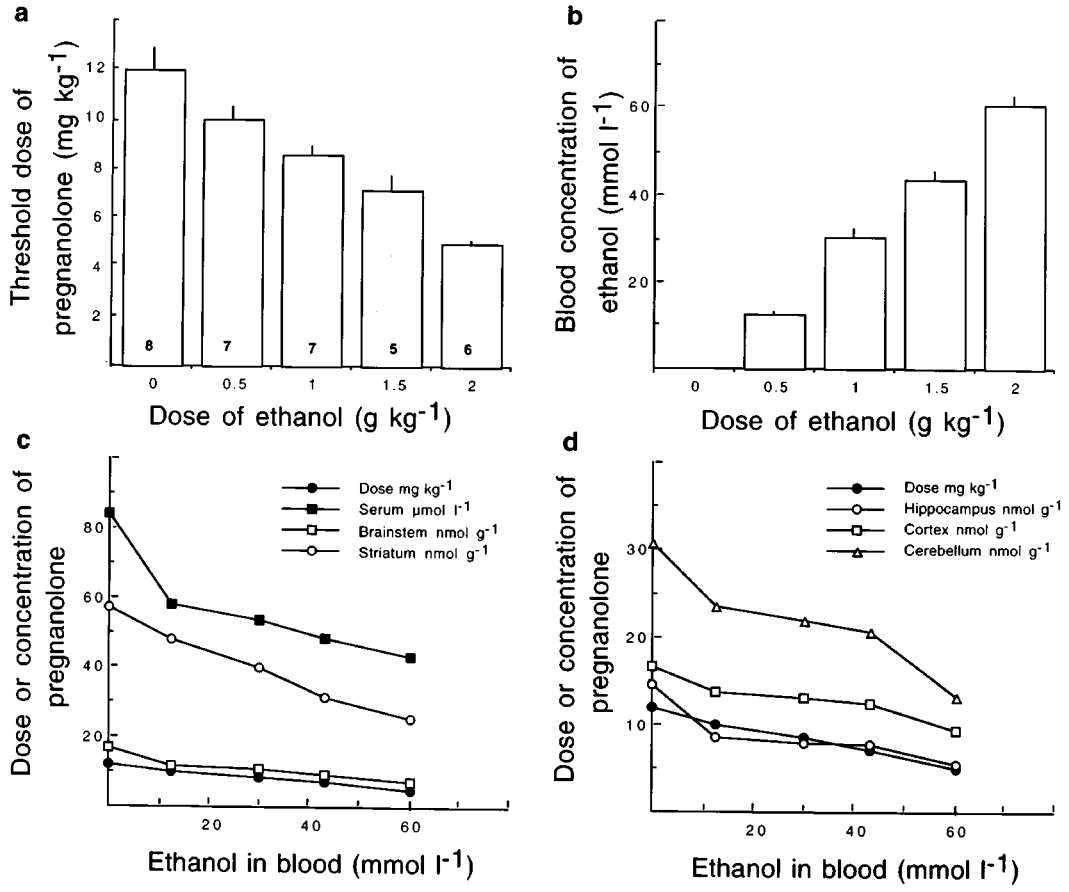

Figure 1 Different effects of ethanol recorded at induction of the 'silent second' (SS) with pregnanolone. *n* is given inside the bars in (a). (a) Effect of different doses of ethanol on the threshold doses of pregnanolone needed to induce the SS. s.e.mean indicated at the top of the bars. (b) Blood concentrations of ethanol obtained at the SS with different doses of ethanol given 15 min before start of induction of anaesthesia with pregnanolone. s.e.mean is indicated at the top of the bars. (c,d) Relation between blood concentrations of ethanol and different tissue concentrations of pregnanolone reached at the SS. For reference the dose of pregnanolone is plotted in both panels. The s.e.mean (μmol l^{-1} or nmol g^{-1}) in the different tissues varied between: 1.2–6.2 in serum, 0.7–1.8 in brain stem, 1.5–7.6 in striatum, 0.7–1.4 in hippocampus, 0.7–1.6 in cortex and 0.8–3.4 in cerebellum.

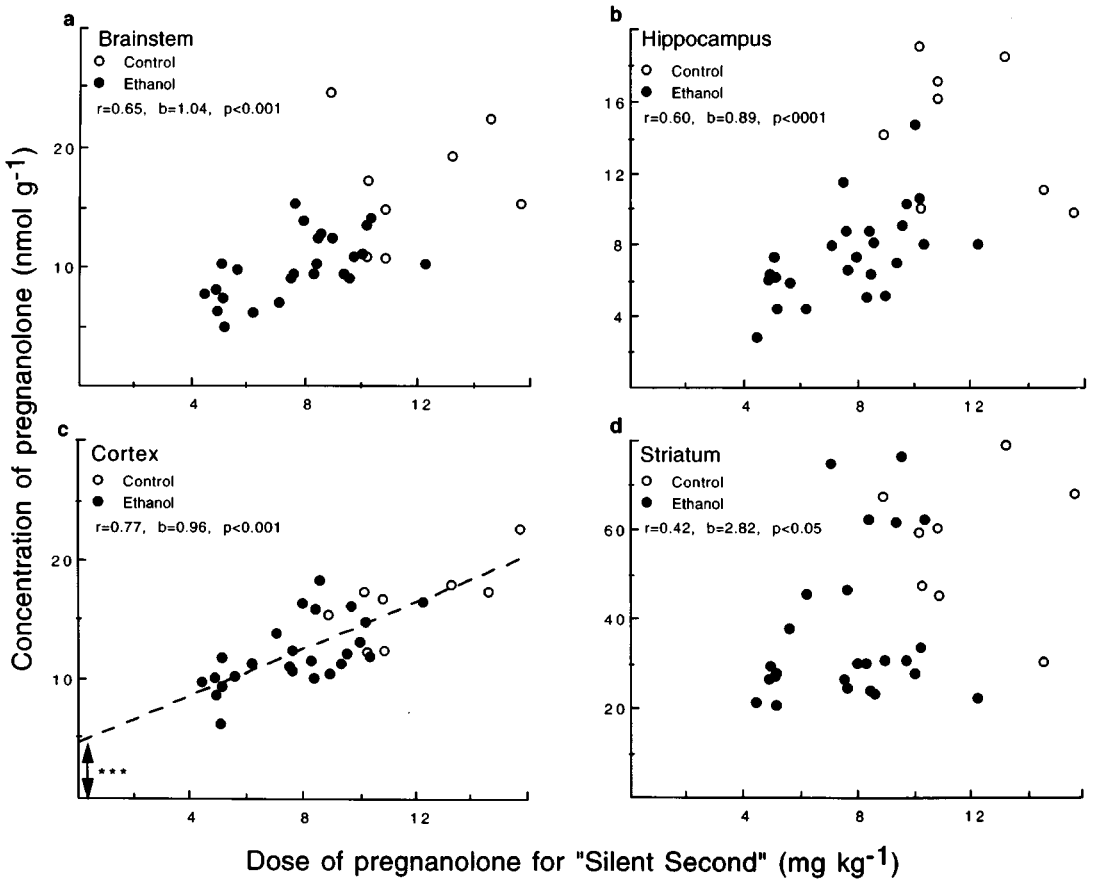

Figure 2 The relationship between the threshold dose of pregnanolone at induction of SS and the concentration of pregnanolone in four different parts of the brain (a,b,c,d). The correlation (r) and regression (b) coefficients and their significance (P) are given in each separate panel.

Ethanol assay

The concentration of ethanol in blood was determined by gaschromatography according to a method described earlier (Wahlström & Widerlöv, 1971) with 1-pentanol as internal standard.

Statistical analysis

All results are presented as means. Errors are given as one standard error of the mean (s.e.mean). Differences between means were tested using Student's t-test. $P<0.05$ in two-tailed or sometimes in one-tailed tests were taken as a significant difference. NS = not significant. Linear parametric regression coefficients (b) and corresponding correlation coefficients (r) were used to study relations between variables. n = number of observations, d.f. = degrees of freedom.

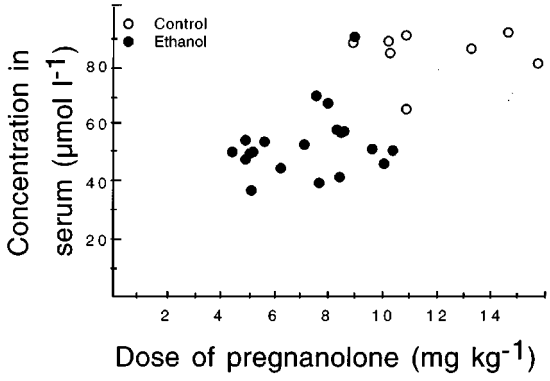

Figure 3 The relationship between the threshold dose of pregnanolone and the serum concentration of pregnanolone at induction of SS.

Results

As expected there were some signs of intoxication after administration of the larger doses of ethanol, but no loss of righting reflex was observed prior to the start of the infusion with pregnanolone. As seen in Figure 1a the different doses of ethanol caused a significant dose-dependent decrease in the dose of pregnanolone needed to induce the SS ($r=-0.81$, $b=-3.16$, d.f. = 31, $P<0.001$). The concentration of ethanol in blood was analysed at the criterion and there was in this case a clear dose dependent significant positive relation with the dose of ethanol ($r=0.99$, $b=30.32$, d.f. = 31, $P<0.001$, Figure 1b). In Figure 1c,d this concentration of ethanol in blood at the SS was used as the abscissa and plotted against the dose and corresponding concentrations of pregnanolone in the serum and different brain tissues. In the serum (Figure 1c) there was an initial large decrease in concentration of

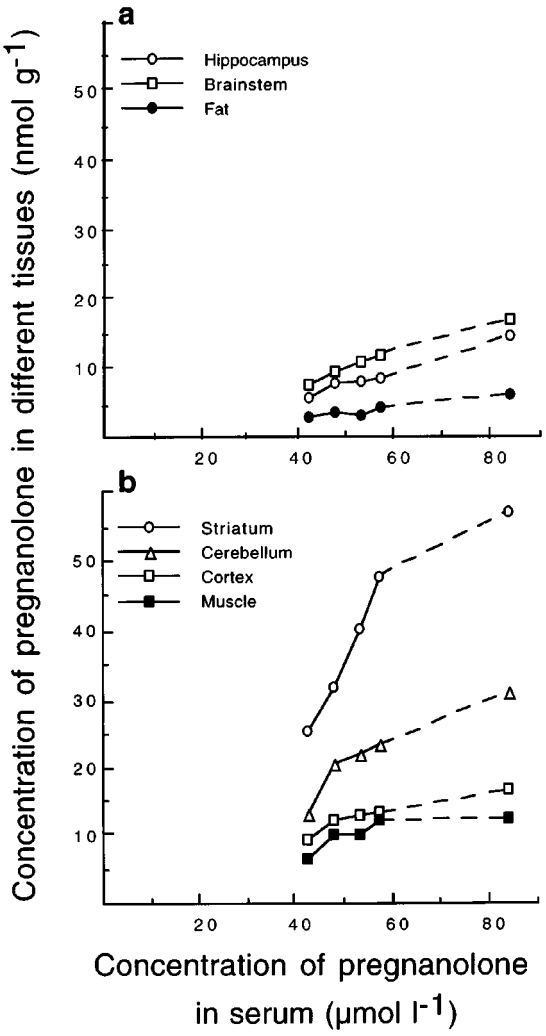

Figure 4 The relationship between the concentration of pregnanolone in the serum and concentrations in different tissues at induction of SS. The tissues are given inside the corresponding panel. s.e.mean are given in the legend to Figure 1c,d. For further details see text.

pregnanolone which did not correspond to a similar decrease in the dose of pregnanolone when plotted either against the blood concentration ($r=-0.84$, $b=-0.11$, d.f.$=31$, $P<0.001$, Figure 1c) or the dose (Figure 1a) of ethanol. After this initial larger decrease there followed a linear decrease in serum concentration of pregnanolone (Figure 1c). An initial larger decrease corresponding to the one seen in the serum was also seen in the concentrations of pregnanolone found in the hippocampus, cortex and cerebellum (Figure 1d) but not in the concentration recorded in the striatum ($r=-0.61$, $b=-0.51$, d.f.$=31$, $P<0.001$, Figure 1c). The change in the concentrations in the brain stem ($r=-0.69$, $b=-0.14$, d.f.$=31$, $P<0.001$, Figure 1c) was more similar to the pattern found in the striatum than the one found in the other three parts of the brain (Figure 1d). In these three parts (hippocampus, cortex and cerebellum) the concentrations of pregnanolone after the initial decrease seemed to be only slightly influenced by the following two doses of ethanol. This is most clearly shown in the hippocampus (Figure 1d) where there is almost no change in concentration of pregnanolone when the concentration of ethanol in blood is increased from 12.68 mmol l^{-1} to 43.41 mmol l^{-1}. However, there were in all brain parts a significant difference between the concentration of pregnanolone between the controls and the one found after the largest dose of ethanol (t-test in all brain parts $P<0.001$, d.f.$=12$) which clearly indicate a pharmacodynamic interaction between ethanol and pregnanolone, but the differences described above indicate a complex pattern of the interaction.

The threshold technique with a criterion founded on a direct measure of a change in the brain activity is, when there are no large external sources of variability, founded on a direct relation between the dose needed to induce the criterion and the concentration at the site of action. This pharmacodynamic aspect is further elucidated in Figure 2 where the dose of pregnanolone needed to induce the SS is plotted against the concentration found in different brain parts at the SS. The data from cerebellum ($r=0.64$, $b=1.92$, d.f.$=31$, $P<0.001$) was omitted since the pattern was very similar to the ones recorded in the brain stem and hippocampus. Thus in all parts of the brain there were the expected positive regression and correlation between the dose of pregnanolone needed to induce the SS and the corresponding brain concentrations. However, differences were also recorded. It is obvious that the variability explained by the correlations differed considerably between the different parts of the brain. In the cortex (Figure 2c) 59% of the variability was explained by the dose while this ratio was 42% in brain stem (Figure 2a), 41% in cerebellum, 36% in hippocampus (Figure 2b) and only 18% in striatum. The regression line on the material from the cortex not only has the smallest variability, but it is also the only one where the cut off on the ordinate significantly differs from zero (4.80 ± 1.31, $P<0.001$, arrows in Figure 2c). When the material in Figure 2c is separated into controls and ethanol treated rats a significant cut off was only retained in the ethanol treated animals (5.54 ± 1.75, d.f.$=23$, $P<0.001$ vs 4.62 ± 4.69, d.f.$=6$, NS). Since it is not possible in any brain part to reach a significant brain concentration of 5 nmol g^{-1} after infusion of 0 mg kg^{-1} of pregnanolone, these data indicate that the ethanol injection beside a pharmacodynamic interaction must also have influenced the pharmacokinetic situation at least in the cortex.

The data given in Figures 1c,d and 2c all indicate that a pharmacokinetic component might be involved in the interaction between ethanol and pregnanolone. Since concentration in the serum is a critical pharmacokinetic variable, the serum data in the present material has been further analysed in Figures 3 and 4. In Figure 3 the relation is given between dose of pregnanolone needed to induce the SS and the corresponding concentration in serum. Figure 3 shows that there was a significant positive relation between the dose of pregnanolone and the concentration of pregnanolone in the serum after injection of ethanol ($r=0.64$, $b=3.47$, d.f.$=23$, $P<0.001$). In the controls no corresponding relation was found ($r=0.02$, d.f.$=6$, NS). This dependence of the serum concentration on the dose of pregnanolone found only after the administration of ethanol explains the rapid decrease in serum concentration of pregnanolone after the lowest dose of ethanol shown in Figure 1c. This initial rapid decrease in the concentration of pregnanolone in the serum after the lowest dose of ethanol also seems to have influenced

the tissue concentrations in at least the hippocampus, cortex and cerebellum (Figure 1d).

The initial rapid decrease of the serum concentration of pregnanolone found in the dose response curve with ethanol, which was not due to a corresponding decrease in the dose of pregnanolone (Figure 1c), must then probably be due to an initially rapid loss of pregnanolone from the serum into some tissue(s). This is analysed in Figure 4. Figure 4 shows the concentrations of pregnanolone analysed in the different tissues plotted against serum concentration of pregnanolone. The controls, which had the largest serum concentration, are found to the right in Figure 4a,b. There seemed to be an approximately linear relationship between the controls and the average values recorded after the four ethanol injections in hippocampus, brain stem and fat (Figure 4a). Calculated on the total material these correlation and regression coefficients were in the brain stem $r=0.66$, $b=0.16$, d.f. $=31$, $P<0.001$, in the hippocampus $r=0.66$, $b=0.15$, d.f. $=31$, $P<0.001$ and in the fat $r=0.71$, $b=0.08$, d.f. $=31$, $P<0.001$. There was no significant deviation from 0 at the intercept between the regression lines and the ordinate ($X=0$) in any of these materials. However, in the hippocampus the concentrations after the first three injections of ethanol are almost at the same level, which was seen already in Figure 1d. Thus the regression data presented here could be founded on a more complex pattern of interaction. This means that there remains only the brain stem and the fat in Figure 4a where the relation between the serum concentrations and the tissue concentrations did not seem to be directly influenced by the ethanol injections. In the tissues shown in Figure 4b there were more dramatic results. In the muscle there was, at the lowest ethanol concentration in the serum (12.68 mmol l^{-1}), approximately the same concentration of pregnanolone as the one found in the controls. In the striatum there was the largest decrease in tissue concentration after the ethanol injections. In the cortex and cerebellum there was a steady apparently linear decrease in tissue concentration when plotted against the serum concentration of pregnanolone in the controls and after the three lower doses of ethanol. In both these restricted materials there was a significant regression (cortex: $r=0.58$, $b=0.10$, d.f. $=25$, $P<0.01$ and cerebellum: $r=0.39$, $b=0.17$, d.f. $=25$, $P<0.05$). Furthermore there were also two corresponding cut off points between these regression lines and the ordinate which were significantly different from 0 (cortex: 7.72 ± 1.88 nmol g^{-1}, $P<0.001$ and cerebellum: 13.95 ± 5.13 nmol g^{-1}, $P<0.05$). Thus in these two tissues there were, after the lowest doses of ethanol, a verified increased tissue concentration of pregnanolone when plotted against the corresponding serum concentrations.

Discussion

The simplest and also the most adequate experimental technique to study the interactions between two drugs *in vivo* is the isobolographic method (Berenbaum, 1989; Gessner, 1995). This method needs a predetermined endpoint in a test system to evaluate the effective dose-combinations of the two studied components. The ED_{50} (dose needed to induce a change in 50% of the tested population) of a number of different combinations of the tested drugs is often chosen (Gessner, 1995) as the way to measure pharmacodynamic interaction, but the precision of such studies is dependent on the number of tests which the experimenter can afford to perform. If both drugs can induce a defined criterion such as the SS used in the present paper the efficiency of the testing can be increased tremendously (Norberg & Wahlström, 1988; Norberg *et al*., 1999). To avoid large differences in the time needed to induce the criterion, which can cause an unwanted pharmacokinetic interference, the initial test of both drugs should be performed at the optimal dose rate and the dose rate at which the mixtures of the drugs is infused should consist of balanced fractions of these optimal dose rates. In such a defined test system different mixtures can give different kinds of interactions (potentiation, additative interaction and antagonistic interaction, Norberg & Wahlström, 1988). This means that tests which have a duration such as anaesthesia times should be avoided, since the mixture of the drugs could change with time if there is a difference in the pharmacokinetic properties of the tested drugs. Despite such precautions it is still possible to have pharmacokinetic components involved in the interaction. Such interferences can be detected at the time when the criterion has been reached if concentrations of the tested drugs are measured at the assumed site(s) of action (Norberg & Wahlström, 1986). Thus to make sure that an interaction is due to pharmacodynamic, pharmacokinetic or combined effects of the two components the *in vivo* test must be combined with determination of the concentration of the used drugs.

The change in the dose of pregnanolone when plotted against blood concentration of ethanol seen in Figure 1c,d is linear with a high correlation ($r=-0.84$). This means that it is possible to estimate the intercept with the abscissa, which in the present experiments is the missing point in the isobologram (see Methods). This concentration of ethanol was found to be 105.9 mmol l^{-1}. Performing calculations on this intercept with the abscissa using the fairly linear data (Figure 1c) also found in the brain stem ($r=-0.69$) and striatum ($r=-0.61$) in Figure 1c gave values of 110 and 109 mmol l^{-1} respectively. Since these intercepts were all very close to each other the value of these intercepts as the estimated point where the hypothetical concentration of ethanol (approximately 108 mmol l^{-1}) needed to induce the SS would have been found, is strengthened. From a toxicological point of view this extrapolation of the blood concentration of ethanol was clearly below the blood level of 202 mmol l^{-1} which can give a respiratory arrest in the rat (Maling, 1970).

The reduction of the doses of pregnanolone needed to induce the SS by the doses of ethanol used in the present experiments (see Figure 1 and the Results section) excludes the possibility of an antagonistic interaction. In the remaining choice between a potentiation or an additive interaction the linearity seen in the data in Figure 1c clearly points to an additive interaction. Such an additive interaction would mean that at the SS 1 mmol l^{-1} of ethanol in the serum reduces the needed concentration of pregnanolone with 0.14 nmol g^{-1} in the brain stem and with 0.51 nmol g^{-1} in the striatum. This interpretation is further strengthened by the close relation between the three intercepts discussed above, but the lack of direct data on the concentration of ethanol needed to induce the SS, when given alone, makes it impossible to eliminate

the possibility of a potentiation if higher doses of ethanol had been investigated. However the present results on circulation obtained already with 2.0 g kg^{-1} (see Discussion below) might seriously invalidate the possibility to interpret data obtained with such higher doses.

Due to the feedback involved in the threshold determination, a linearity in the reduction of the dose of pregnanolone (Figure 1a,c) should correspond to a similar linearity in the concentration from the tissue were the site of action is situated. Such a linearity was found in the data from the brain stem and the striatum (Figure 1c), which point at these two regions as the possible site(s) of the interaction. When dose of pregnanolone is used as the abscissa (Figure 2) it is quite clear that there is a stronger relation in data from the brain stem (Figure 2a) than in data from the striatum (Figure 2d). This points at the brain stem as the primary site of action. This conclusion is strongly supported by a similar conclusion in an earlier study (Zhu *et al*., 2001) where the pharmacokinetic and pharmacodynamic properties of allopregnanolone and pregnanolone at induction of SS with different dose rates were investigated in male SPRD rat of the same age as in the present investigation. The same brain parts were also used in the analyses of concentrations of allopregnanolone and pregnanolone found at SS. The study (Zhu *et al*., 2001) revealed that the brain sensitivity to both steroids was similar and that in the cortex, striatum and cerebellum there was a significant increase of steroid concentrations as the dose rate was increased while the steroid concentrations in brain stem and hippocampus remained unchanged. Assuming that there should be no change in sensitivity due to change in dose rate these two brain parts could be the primary sites of action. The case for the brain stem as a primary site of action was further strengthened in the previous investigation (Zhu *et al*., 2001) by the fact that a significant correlation between dose of pregnanolone and concentration in the tissue was only found here. Thus the possibility that the primary site of action of pregnanolone and allopregnanolone in inducing anaesthesia in the brain stem is supported by the present results, which means that the primary site of action generating the pharmacodynamic interaction between ethanol and pregnanolone could also be located in the brain stem. This pharmacodynamic interaction is probably additive.

One possible pharmacokinetic interaction is the large reduction in serum concentration of pregnanolone seen after the lowest does of ethanol (Figure 1c), which could not be explained by a similar reduction in the dose of pregnanolone. The difference between the ethanol treated rats and the controls in Figure 3 pointed to an increased loss of pregnanolone in some tissue(s) induced by low concentration of ethanol. Of the analysed tissues there were reduced tissue concentrations in hippocampus, brain stem, fat, striatum, cerebellum and cortex (Figure 4a,b) after the lowest dose of ethanol when compared to the controls. However, this was not the case in the muscle where the same concentration of pregnanolone was found in both groups, despite the large reduction in the serum concentration (Figure 4b). Among the other tissues the smallest decrease was found in cortex. Thus one certain candidate for a relative increase in uptake of pregnanolone to explain the increased loss from the serum after low ethanol doses is the muscle tissue with cortex as a close candidate but a number of other tissues were not analysed.

In three brain parts (hippocampus, cortex and cerebellum, Figure 1d) there was a reduced uptake of pregnanolone after the highest dose of ethanol with no corresponding change in serum concentration (Figure 1c). This indicates a local change, where one alternative could be a reduced blood flow (Altura & Altura, 1996). Goldman *et al*. (1973) reported reduced blood flow in conscious rats in cerebellum and hippocampus at blood concentrations of ethanol between 32–54 mmol l^{-1}. In dogs Friedman *et al*. (1984) showed that blood levels of ethanol around 48 mmol l^{-1} markedly reduced the blood flow in cortex but had no effect in hippocampus or brain stem. Direct studies of cortical arterioles *in vivo* by Altura *et al*. (1983) showed an ethanol induced vasoconstriction, which was clearly dose dependent. These data indicate that the change in uptake of pregnanolone found in cortex, cerebellum and hippocampus at blood levels above 43 mmol l^{-1} (Figure 1d) in the present experiment could be due to a reduced blood flow in these areas. This clearly is a pharmacokinetic change induced by ethanol which can have unpredictable consequences on the effect of pregnanolone, other steroids and drugs.

Since ethanol can influence a number of processes in the cell membrane (Deitrich *et al*., 1989; Eckardt *et al*., 1998), an important pharmacokinetic component is the relation between the serum concentration and the corresponding tissue concentration. Figure 4 shows that this relation between ethanol and pregnanolone is certainly not a simple one. To elucidate this important issue the Q values (tissue concentration/serum concentrations) are given in Table 1. As seen already in Figure 4 there was a large variability in the concentration data between different brain parts. This is of course retained in the Q values, which in the ethanol treated groups are given as a difference from the corresponding control values. Table 1 reveals interesting differences in the patterns found between the different brain areas. In the

Table 1 Change from controls induced by ethanol on the relation between brain tissue and serum concentrations of pregnanolone (Q) after induction of SS in male rats

	Dose of ethanol (g kg^{-1})				
Tissue	*0.5*	*1.0*	*1.5*	*2.0*	*Controls* (±s.e.mean)
Cortex	+0.047*	+0.055**	+0.062**	+0.022	0.197±0.014
Cerebellum	+0.054	+0.073	+0.055	−0.061	0.369±0.035
Striatum	+0.261	+0.100	+0.004	−0.088	0.683±0.064
Brain stem	+0.015	+0.009	−0.008	−0.030	0.202±0.020
Hippocampus	−0.019	−0.021	−0.017	−0.046	0.175±0.019

*$P<0.05$, **$P<0.05$ (one sided).

ethanol treated groups the lowest Q value was in all brain parts recorded after the dose of 2 g kg^{-1}. In the hippocampus the Q value was lower (a small uptake into the tissue?) than in the controls after all ethanol doses. Very small changes compared to the controls were seen in the brain stem. In the cortex, where the only significant changes from the controls were recorded, the Q-value was increased with an exception after the largest dose of ethanol. This clearly strengthens the impression from Figure 2 that concentration of pregnanolone in cortex is regulated in a different manner than the concentration in other brain areas. This difference could be due to difference in uptake and/or elimination. An increased endogenous production is also an alternative although this possibility is limited by the fairly short times involved.

The interaction between ethanol and pregnanolone established in this investigation support the hypothesis that some of the effects of ethanol can be replaced by neurosteroids (Morrow *et al*., 1999). The present data are also in line with the findings that ethanol and neurosteroids are exchangeable in behavioural discrimination tests (Grant *et al*., 1996; Bowen *et al*., 1999) and the enhancement of anaesthesia time found in mice (Melchior & Allen, 1992). However the present more detailed analysis of the interaction revealed a complex pattern which to some extent was dependent on the dose of ethanol. From a kinetic point of view the results from cortex are interesting when compared to recent data obtained on the effect of ethanol on the concentration of allopregnanolone in cortex (VanDoren *et al*., 2000). After i.p. administration of 3.5 g kg^{-1} ethanol there was, in rats killed at the end of the anaesthesia time, a steady increase in the concentration of allopregnanolone in cortex ($r=0.59$ d.f. $=35$, $P<0.001$). This result was interpreted as an indication that allopregnanolone contributed to the anaesthetic effect of ethanol. Since the ethanol concentration at the longest durations of anaesthesia (100–200 min) probably had decreased to the levels used at induction in the present experiment and since pregnanolone and allopregnanolone seem to be very similar as far as induction of anaesthesia is concerned (Zhu *et al*., 2001) it is unlikely that the low concentrations in cortex (3–14 ng g^{-1}) reached in experiments performed by VanDoren *et al*. (2000) could have contributed to any large extent to the anaesthesia which in the present experiments needed around 300 times larger concentrations. However, the positive correlation found by VanDoren *et al*. (2000) is interesting in relation to the kinetic situation in cortex which was discussed above. Could allopregnanolone be retained in the cortex by ethanol in a similar manner as pregnanolone?

In a recent review (Falkenstein *et al*., 2000) five receptor systems are listed at which neurosteroids could be active. When dealing with ethanol the list is even longer (Deitrich *et al*., 1989; Eckardt *et al*., 1998) and to this list can be added neurosteroids (Morrow *et al*., 1999). As stated in the introduction the $GABA_A$ system is a very strong candidate as the site where ethanol and neurosteroids could interact. In this complex system (Lambert *et al*., 1995; Mehta & Ticku, 1999) both neurosteroids and ethanol have well established effects and it is very likely that this is the system which is responsible for the dynamic part of the interaction found in the present investigation. Although attractive to explain an additive interaction the increase in allopregnanolone concentrations in the cortex (VanDoren *et al*., 2000) induced by ethanol does not seem to be large enough to substitute for the reduction in concentration of pregnanolone recorded in the present experiments. Thus the working hypothesis for further analysis of the documented interaction between ethanol and pregnanolone is a common pathway through the $GABA_A$ system. Since brain stem seems to be a critical area more detailed knowledge of the $GABA_A$ system in this part of the brain also seems to be needed.

The use of a threshold dose founded on a relevant criterion combined with analysis of drug concentrations has a number of advantages in interaction studies compared to the more traditional dose response concept. Since the individual sensitivity is directly measured as the threshold dose, changes in this sensitivity could be measured in interaction studies simply by recording changes in the threshold dose. As seen from the present and earlier experiments (Norberg & Wahlström, 1986) a combination with analysis of the relevant drug concentrations in different tissues improves the possibility to separate this sensitivity into both pharmacokinetic and pharmacodynamic components of the interaction. The present analysis of the interaction between ethanol and pregnanolone strongly supports the concept of an additive interaction between these substances when induction of anaesthesia is studied. Founded on the present knowledge of the site of action of these two drugs, it is very likely that the source of the additive interaction is the $GABA_A$ system. Furthermore the present experiment supports earlier results from this laboratory that the brain stem is a critical brain tissue involved in the induction of anaesthesia with pregnanolone. There was no indication of a change in the pattern of the pharmacodynamic interaction depending on the dose of ethanol. However this was clearly the case when dealing with the pharmacokinetic interaction. At low doses (0.5 g kg^{-1}) the threshold dose of pregnanolone was reduced. All tissues except the muscle also had a reduced concentration of pregnanolone. Founded on the pattern of different changes in the different tissues it is possible to conclude that serum is the primary contributor to this retained high concentration of pregnanolone in the muscle but the brain parts were not equally influenced by the rapid loss from the serum. At high doses of ethanol (2.0 g kg^{-1}) there was also a different pattern of pregnanolone concentration in different tissues. The serum concentration was reduced as expected from an additive interaction but the decrease in cerebellum, cortex and hippocampus was larger than expected. Due to the uninfluenced serum concentration a reduced blood flow in these brain parts was suspected. The concentration of pregnanolone in cortex is certainly not regulated in the same manner as in other brain parts. The reason for this increased concentration seen both when related to the dose and the serum concentration of pregnanolone is uncertain. An increased uptake is the most likely explanation in the present experiment with a continuous infusion of pregnanolone but a reduced elimination or an increased endogenous production cannot be excluded.

We thank Mrs K. Wahlström, Mrs L. Gustavsson and Mrs A. Andersson for their skilful technical assistance. This study was supported by the Medical Research Council project No. 4X-11198, a 'Spjutsspets' grant from Umeå Sjukvård, an EU-regional grant and grants from the Faculty of Medicine and Dentistry, Umeå University, 'Folkhälsoinstitutet', and 'Systembolagets forskningsfond'.

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(Received July 3, 2001
Revised September 5, 2001
Accepted September 11, 2001)