Some effects of prenatal exposure to aluminium in mice *(Mus musculus)*.

by

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I hereby declare that this thesis was composed by myself and is my own work except where acknowledged.

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ABSTRACT

The influence of prenatal exposure to aluminium sulphate ($Al_2(SO_4)_3$) on the behavioural development of mice (*Mus musculus*) from two inbred strains was examined. Pregnant CBA/T6 and C57BL/6J mice were exposed by intraperitoneal (i.p.) injection (200mg/kg) or orally (750, 1000 and 1250mg/L) to $Al_2(SO_4)_3$. On postnatal day one, pups were cross-fostered and tested in a variety of ethological measures, from birth to adulthood, to assess effects on the mother and the behavioural development of the pups. As a neurochemical marker for the cholinergic system, choline acetyltransferase (ChAT) activity was measured at different developmental stages.

Breeding performance, the length of gestation and sex ratio were unaffected by Al exposure administered via the i.p. route. There was a transient reduction in maternal weight gain during gestation. CBA pups born to Al-treated mothers exhibited lower body weights at birth; this reduction persisted into adulthood only in treated pups reared by treated mothers and was more pronounced in the case of female mice. The body weight changes were accompanied by delays in the maturation of several of the tests of sensory-motor development. Al-treated CBA females were hypoactive at weaning compared to controls whilst the converse was true for males. 77% control and 55% treated males reached criterion in a maze test and controls required fewer days to do so. The effect of Al exposure on the cholinergic system was dependent on the region of the brain studied, and still showed significant effects in the adult.

In utero exposure to injected Al resulted in a reduction in the rate of ultrasonic calling by CBA pups and was accompanied by a delay in the timing of peak calling. C57 pups were not affected to the same degree. Exposure to oral Al caused a similar but less obvious trend towards a diminished calling. The inclusion of the recording of ultrasonic calling is recommended in any test battery aimed at assessing behavioural teratogenicity.

Only slight changes to certain components of maternal behaviour were observed following exposure to Al via the i.p. route. Al-exposed CBA females spent less time *nursing* and were more involved in non pup-directed behaviours. Conversely, C57 females showed enhanced *nursing*. Thus, the alterations to pup development are unlikely to have resulted from any disruption to maternal behaviour.

The route of administration proved to be an important factor in determining the toxicity of $Al_2(SO_4)_3$ and the effects of oral exposure were dose dependent. Orally exposed CBA pups had lower birth weights than controls but showed few further developmental or behavioural deficits. At the 750mg/L dose C57BL/6J pups reared by treated mothers were the heaviest throughout the experiment but at the highest dose (1250mg/L) the converse was true. More treated males completed the maze and were less affected by a scopolamine challenge than controls. The effect of oral Al on ChAT activity was sex, strain and treatment group dependent. In general, the trend within CBA males, females and C57 females was towards a decrease in ChAT levels following oral Al exposure. The trend within C57 males was more variable.

These results demonstrate behavioural and neurochemical alterations in the offspring of mice exposed to aluminium during gestation. Further, the effects of such exposure are also present in the adult animal suggesting persistent changes to brain and behaviour following prenatal exposure. Such findings have serious implications for the currently assumed lack of risk associated with exposure to aluminium.

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CHAPTER 1

1.1 GENERAL INTRODUCTION

Research over the last 50 years has shown that the human placenta does not provide an impermeable barrier to the entry of foreign agents and that many harmful substances can readily cross it, leading to accumulation within the foetus.

The foetus is unable to metabolise or excrete substances as efficiently as the adult due to the immaturity of these pathways (Hammarström, 1966; Erikson and Yaffe, 1973; Spyker, 1975; Weaver, Laker and Nelson, 1984; Mirmiran and De Boer, 1988). The human foetal brain is also accessible as the blood-brain barrier is not fully developed until the end of the first year of life (Statz and Felgenhauer, 1983). Since human brain development continues into infancy, noxious chemicals which can pass into the milk may continue to place the neonate at risk. Thus, prenatal exposure to chemicals can affect the developing foetus directly and the neonate may continue to be affected via the mother's milk. In an environment increasingly contaminated with chemicals, the developing foetus and neonate are particularly at risk.

1.1.1 Background to drug testing

The vulnerability of the developing human foetus has been highlighted by two disastrous events. Although research into the potential of certain substances to cause malformations in the foetus was under way by 1960 (see review by Wilson, 1979), it was not until the thalidomide tragedy (McBride, 1961; Lenz, 1962) that the susceptibility of the developing foetus was fully realised. During this

incident many of those pregnant women prescribed the drug during the first trimester of pregnancy (Lenz and Knapp, 1962), gave birth to children with severe congenital physical deformities (phocomelia). This event emphasised the need, and was undoubtedly the impetus, for vigorous and controlled experimentation of substances before their release into general circulation. The second incident occurred in Japan and involved the ingestion by pregnant women of food contaminated with methylmercury. The children of these mothers developed neurological and behavioural abnormalities as a result of this exposure (Takeuchi, Eto and Eto, 1979). The disease which resulted from this incident of methylmercury poisoning has become known as the Minamata disease and resulted from the disposal of industrial waste in the local river which was sequestered by the fish population and thus entered the food chain.

Over the same period, animal experiments prior to 1960 had shown that congenital malformations could be produced in the foetus following exposure of the mother to diets deficient in vitamins (Warkany and Nelson, 1940), to viral infection e.g. the rubella virus (Gregg, 1941, cited in Wilson, 1979) or to radiation (Wilson, Jordan and Brent, 1953). Hence it was realised that exposure of the pregnant animal to certain substances could alter intrauterine development and lead to malformations within the foetus. Thus, the field of teratology i.e. the study of substances which can cause overt morphological deformities, developed.

It is known that a considerable proportion of congenital anomalies are genetic in origin and this may also be true for behavioural abnormalities e.g. mental retardation which forms the largest category of defects among children and adults (Kopp and

Parmlee, 1986). However, the majority of behavioural anomalies have an unknown aetiology and thus it is plausible that some may result from teratogenic causes (Wilson, 1973).

Alterations to brain development can have far reaching consequences on subsequent behavioural development even in the absence of visible morphological defects (Ornoy and Yanai, 1980). For this reason, testing of potential teratogens in more recent years has considered doses much lower than those required to produce obvious morphological alterations within the developing foetus, but which may result in longterm subtle behavioural consequences. This sub-discipline has been called "behavioural teratology" (Werboff and Gottlieb, 1963).

1.1.2 Behavioural teratology

The CNS of the developing foetus is especially susceptible and is affected at doses far lower than those required to produce changes in the adult CNS (Kornetsky, 1970; Vorhees and Butcher, 1982; Riley and Vorhees, 1986). For example, exposure of four-day old Sprague-Dawley rats to cadmium chloride (4mg/kg body weight) by subcutaneous injection, resulted in lesions within the cerebral cortex, cerebellum and caudate-putamen of the neonatal brain and an increase in motor activity (Wong and Klassen, 1982). No such effects were found following exposure of eight-week old adults to the same treatment. The recognition of this point has stimulated research into postnatal effects of prenatal exposure to agents which can traverse the placenta. This has led to the identification of a wide range of substances in our modern environment which can act as

behavioural teratogens. Sette (1987) estimates that there are at least 800 behavioural teratogens, including certain drugs, various food additives and contaminants and a range of industrial chemicals.

Perhaps one of the first behavioural teratogens to be identified was alcohol. In 1973 Jones and Smith described a number of anomalies in the offspring of alcoholic mothers which included growth deficiency, characteristic facial changes and mental retardation. These abnormalities have collectively become known as the "Foetal Alcohol Syndrome" (FAS). Since its initial description it is estimated that the worldwide frequency of FAS is 1.9 in every 1000 births (Abel and Sokol, 1987). Similarly, identification of the Foetal Hydantoin Syndrome resulted from the recognition of several abnormalities, including facial anomalies, limb defects and growth retardation, in children born to mothers who were prescribed anticonvulsants during pregnancy (Hanson and Smith, 1975).

During this century social change has resulted in many more working women, so that their risk of occupational exposure to potentially harmful substances is great. Behavioural teratology has emerged as a prolific area of scientific research in response to the ever increasing number of new chemicals, and the levels of such substances, entering the environment and workplace. Between 200 and 1000 new chemicals are introduced into the marketplace every year (Hemminki and Lindbohn, 1988), the behaviour of many of which is not fully understood, thus increasing the risk of significant human exposure. Hulebak (1987) estimated that 7.7 million American workers are exposed to at least 1 of the 67 substances which the National Institute for Occupational Safety and Health has recommended to be controlled.

1.1.3 The need for animal studies

Unfortunately in a number of cases there has been no recognition of potential risks to public health until a major human disaster has occurred, and animal studies have only been initiated afterwards. Further, in humans it is difficult to relate subtle behavioural consequences of prenatal exposure to adverse influences which may not become apparent until many years after birth. Since little is known at present about the effects of many chemicals in the environment on human health and, in particular, on the developing foetus, the development of animal models enables us to address specific questions. The need for adequate and reliable screening of new substances warrants urgent research to prevent further unnecessary exposures.

Many test batteries have been recommended for the assessment of animals exposed to toxicants (Spyker, 1975; Buelke-Sam and Kimmel, 1979; Zbinden, 1981; Vorhees and Butcher, 1982; Pryor, Uyeno, Tilson and Mitchell, 1983; Tilson and Mitchell, 1984; Adams, Buelke-Sam, Kimmel, Nelson, Reiter, Sobotka, Tilson and Nelson, 1985). Their underlying principle is that a variety of behaviours should be assessed, as it is unlikely that all possible toxicants will affect the same behaviour. Thus, any test battery should be reliable, reproducible and sensitive to even the most subtle of effects.

The aim of the original project undertaken in this laboratory was to develop a rapid and cheap screen for potential behavioural teratogens. A number of tests were incorporated into the screen to

monitor a wide variety of behaviours. Reliability of this test battery in detecting developmental toxicity was established using the known behavioural teratogen phenobarbital (Sedowofia, Innes, Peter, Alleva, Clayton and Manning, 1989). In this thesis the test battery has been used to investigate the behavioural teratogenicity of aluminium, a ubiquitous element which has been implicated in a number of human pathological conditions but whose effects on reproduction and teratogenic potential are not known.

1.2 BEHAVIOURAL TERATOLOGY TESTING

The main experimental variables which must be considered in the design of a behavioural teratological experiment are essentially the same as those which govern all experiments in teratology. For this reason examples will be drawn from both disciplines. These variables are outlined below:-

i. Stage of gestation at exposure

The period over which an animal is exposed to an insult determines the type of change which results. The importance of this principle was recognised as long ago as 1931 when Stockard noted that the induction of certain types of malformation was dependent on the time of exposure (cited in Wilson, 1979). In the human, Lenz and Knapp (1962) have estimated that the critical stage of pregnancy during which exposure to thalidomide results in physical deformities, is between postconception days 27-40.

It is considered that in the rodent exposure to an agent during the first or third week of gestation is without effect, but that the second week represents a time of vulnerability as it is during this stage that organogenesis occurs (Coyle, Wagner and Singer, 1976; Vorhees and Butcher, 1982; Kutscher and Nelson, 1985). Although this is generally true exceptions can be found. For example, the exposure of pregnant Wistar rats to vitamin A on gestation days 17 and 18, following completion of organogenesis, resulted in learning deficits (Hutchings and Gaston, 1974).

Prenatal exposure of Sprague-Dawley rat pups to methylmercury (8mg/kg) on day 8 of gestation, increased the duration and decreased the frequency of ultrasonic calling during postnatal days 4-8. No differences were found if the same dose was administered on day 15 of gestation (Cagiano, Cortese, De Saliva, Renna and Cuomo, 1988).

ii. Route of administration

Toxic effects of substances may be altered markedly by the means by which they are introduced into the subject. For example, Kimmel (1977) exposed pregnant CD rats to disodium ethylenediamine tetraacetic acid (EDTA), a chelating agent, either in the diet, by gastric intubation or by subcutaneous (s.c) injection during days 7-14 of gestation. EDTA present in the diet did not result in any maternal deaths but did cause severe maternal toxicity; following gastric intubation three quarters of the exposed mothers died and EDTA proved to be lethal to some pregnant dams upon s.c injection (Kimmel, 1977). Conversely, EDTA caused a high

incidence of malformations within the offspring of dams exposed via the diet, fewer malformations if exposure was by gastric intubation and no signs of offspring malformations following administration by s.c injection (Kimmel, 1977).

iii. Dose

It may be presumed that any substance has the potential to be teratogenic if it is administered at a sufficiently high dose. It is not the aim of behavioural teratologists to use doses which result in physical malformations but rather to test a range of doses to assess their ability to cause more subtle behavioural anomalies. Hughes and Annau (1976) administered methylmercury (MeHg) hydroxide by intubation to pregnant CFW mice at a dose of 0, 1, 2, 3, 5 or 10mg/kg body weight, and found that the litter size decreased as a function of dose. The mean number of pups per litter was 8.55($\pm$0.71) following exposure to methylmercury hydroxide at 1 mg/kg, compared to a mean of 2.53($\pm$0.94) after the 10mg/kg dose (Hughes and Annau, 1976).

Exposure of Sprague-Dawley rats to MeHg at a dose of 0.25, 1.25, 2.5 or 5.0mg/kg by gavage on gestation days 6-15, resulted in a delay in surface righting and an increase in the number of central squares entered by subjects exposed to the 2.5mg/kg dose only (Geyer, Butcher and Fite, 1985).

iv. Chemical nature of the agent

The effects of exposure to a substance is determined by its chemical speciation. Exposure of pregnant Sprague-Dawley rats to valproic acid, an anticonvulsant commonly used in the treatment of epilepsy, at a dose of 300mg/kg during days 7-18 of gestation, resulted in reduced maternal and foetal weight and an increase in offspring malformations. When valproate was administered as *trans*-2-ene valproic acid no such effects were seen (Vorhees, Acuff-Smith, Weisenburger, Minck, Berry, Setchell and Nau, 1991). The absorption of mercury (Hg) is increased when it is in an alkylated form. Kutscher and Nelson (1985) have estimated that the absorption of Hg from the rat gut is 0.01%, 15% and 18% of the delivered dose for liquid Hg, inorganic Hg and MeHg respectively.

v. Species susceptibility

The thalidomide tragedy highlighted a major consideration for testing potential teratogens i.e. species differences in susceptibility to a substance, and it provides one of the most striking examples. Research into thalidomide was carried out only with rats which proved to be insensitive to its effects, whilst exposure of primates and rabbits can result in the characteristic phocomelia. Pharmacogenetic factors e.g. differences in rates of uptake, metabolism and excretion, are responsible for such differences (Clayton, 1980; Clayton and Zehir, 1982; Yanai, 1983; Shuster, 1990). However, not only have studies investigating the effects of an agent on the developing foetus found large variations in the response

of different species, but there are also considerable differences between strains of the same species. For example, following prenatal exposure to methylmercury (10mg/kg) on day 14, 15, 16 or 17 of gestation, 67, 88, 75 and 48% of B10.D2 mice offspring were found to have developed hydrocephalus whereas offspring from the C57BL/6 inbred strain developed hydrocephalus following day 15 exposure only and offspring from the DBA/2 strain never developed it (Inouye and Kajiwara, 1990). Finnell, Shields, Taylor and Chernoff (1987) found the LM/BC inbred mouse strain to be more sensitive to the effects of gestational oral administration of phenobarbital (240mg/kg) than the C57BL/6J strain. 47% of LM/BC foetuses exhibited congenital malformations compared to 29% within the C57BL/6J strain (Finnell, *et al.*, 1987).

In addition to the above variables, the effect of early environmental influences on postnatal development suggests further design requirements for good experiments.

vi. Fostering

The influence of the mother on postnatal pup development has proved to be a confounding variable in behavioural teratology experiments. Any alteration to the hormonal, nutritional or behavioural status of the mother following prenatal drug treatments, may alter her ability to maintain or initiate adequate maternal care, which will have consequences for the development of the offspring. In order to differentiate between the direct effects of a toxicant upon the developing foetus from indirect postnatal effects arising from

continued transfer to the neonate via the dam's milk, a fostering procedure should be included in the experimental design. Many authors have recommended this (Spyker, 1975; Coyle, Wagner and Singer, 1976; Hutchings, 1985; Chiarotti, Alleva and Bignami, 1987; Tilson, 1987), whilst others have questioned the validity of studies which have not included such a procedure (Abel, 1978; Tucker, 1985).

vii. Characteristics of the litter

The litter in itself is a unit and a pup's behavioural development can be influenced by a number of factors relating to the characteristics of the litter in which it is reared. Thus, a pup can not be considered in isolation and the effect of such variables on development has been recognised as a source of variation which should be controlled for (Kimmel, Buelke-Sam and Adams, 1985; Chiarotti, Alleva and Bignami, 1987). A further advantage of incorporating a fostering procedure, especially one involving cross-fostering during which pups are distributed between experimental groups, is that such effects are reduced (Ulbrich, Schreiner and Bass, 1985).

Further, the number of offspring present in a litter should be kept constant to even out litter effects and to avoid any nutritional stress.

1.3 WHY THE INTEREST IN ALUMINIUM?

Aluminium (Al) is the third most abundant element in the earth's crust. It is ubiquitous in nature and because of its high reactivity and amphoteric properties it is usually found in combination with other elements especially oxygen. Al is not thought to have a biological function although it has been suggested that man has a definite need for it for normal metabolism (Sorenson, Campbell, Tepper and Lugg, 1974). For a long time it was presumed that exposure to Al was without toxic effect. As a result of this assumed safety, Al has become widely used in the food and food processing industries, in cookware, medicine, cosmetics and as a flocculant in water treatment, all of which increase the daily intake of Al. Greger (1985) estimates the daily adult Al intake to be 20-40mg Al/day. This compares to an intake of between 840-5000mg following antacid ingestion (Lione, 1983). From these diverse routes of contact, exposure to Al is unavoidable. However, in contrast to this assumed harmlessness, Al has, in more recent years, been implicated as a causative factor in several human diseases.

The lack of concern over the effects of Al ingestion probably resulted from the assumption that it was not absorbed from the gastrointestinal (GI) tract, and under normal conditions any Al which did enter the body would be effectively excreted by the kidneys (Lote and Saunders, 1991). It is now known that this is not the case and that Al can be absorbed both in rats, following ingestion of aluminium chloride ($AlCl_3$) (Berlyne, Yagil, Ben Ari, Weinberger, Knopf and Danovitch, 1972; Bowdler, Beasley, Fritze, Goulette, Hatton, Hession, Ostman, Rugg, and Schmittdiel, 1979;

Commissaris, Cordon, Sprague, Keiser, Mayor and Rech, 1982) and in humans e.g. from the ingestion of Al containing antacids used to prevent hyperphosphatemia in dialysis patients and in the treatment of gastric ulcers (Kaehny, Hegg and Alfrey, 1977; King, Savory and Wills, 1981). Moreover, the absorption of Al from the gut is known to be influenced by the presence of certain factors. Mayor, Keiser, Makdani and Ku (1977) found the Al concentration in muscle, brain and bone of rats fed a diet of rat chow containing 0.1% $AlCl_3$ and injected with parathyroid hormone, to be greater than in rats fed only the diet. This is of importance for patients on dialysis where high levels of parathyroid hormone are common.

Penetration of the body's barriers by Al has also been found to be facilitated by the presence of certain dietary factors: Slanina, Frech, Ekström, Lööf, Slorach and Cedergren (1986) have reported elevated blood Al levels in normal human subjects following ingestion of aluminium hydroxide ($Al(OH)_3$) in the presence of citric acid. Similarly, Kruck and McLachlan (1989) found that three days following the administration of aluminium maltolate, commonly used in the food processing industry, the plasma Al level was still elevated in treated animals compared to controls. Moreover, possible sites and mechanisms of absorption of Al from the GI tract have been proposed (Savory and Wills, 1991). This evidence suggests that Al can indeed be absorbed and that this absorption is augmented by the concomitant ingestion of certain substances in common use.

Al has been found to interfere with normal levels of some elements which perform essential functions within the body. A phosphorus-depletion syndrome involving such symptoms as

hypophosphatemia and osteomalacia (demineralisation of bone) has been correlated with the chronic ingestion of antacids (Bloom and Flinchum, 1960; Ondreicka, Ginter and Kortus, 1966; Lotz, Zisman and Bartter, 1968). Antacids have also been found to decrease the absorption of fluoride (Spencer, Kramer, Norrie and Wiatrowski, 1980), which has further consequences for bone. Al combines competitively with calmodulin affecting calcium (Ca) regulation (Siegel and Haug, 1983) and Ca dependent processes e.g. within the heart which is regulated by Ca dependent channels.

Another possible route of entry to the human body is through the respiratory tract. Evidence for this has accumulated from the results of studies investigating the significance of industrial exposure to Al. McLaughlin, Kazantzis, King, Teare, Porter and Owen (1962) reported increased Al content in the lungs and brain of workers in an Al powder factory. Further, workers exposed to McIntyre powder (consisting of aluminium oxide), used in Canada up to 1979 to prevent lung disease arising from exposure to silica, did not score as well on tests of cognitive function as did unexposed workers (Rifat, Eastwood, Crapper McLachlan and Corey, 1990).

During the last 20 years research involving Al has focused on its neurotoxic actions. Possible Al neurotoxicity was described as long ago as 1897 when Dölken found that injected aluminium tartrate produced neuronal degeneration in rabbits (cited in Boegman and Bates, 1984). However, it was not until more recently that Al's role as a potential neurotoxin was fully recognised. In their extensive review article concerning Al's role in the environment and human health, Sorenson, Campbell, Tepper and Lugg in 1974 concluded that :-

".... there is no need for concern by the public or producers of aluminium or its products concerning hazards to human health derived from well established and extensively used products."

Within two years of this publication a new clinical syndrome, dialysis encephalopathy which linked Al to a fatal dementia, had been described (Alfrey, LeGendre and Kaehny, 1976). In 1976 Alfrey, LeGendre and Kaehny found an increase in the Al content of brain gray matter of patients with dialysis dementia. This finding was supported by Dunea, Mahurkar, Mandani and Smith (1978) who reported an increased incidence of this condition in patients on long-term dialysis after a change in the method of water purification, which resulted in higher levels of Al. Again, postmortem autopsies revealed a raised brain Al content. This condition is now recognised as dialysis encephalopathy, i.e. a progressive, fatal dementia associated with long-term dialysis treatment (Alfrey, *et al.*, 1976).

In natural populations, a similar form of dementia has been described from regions where there are high levels of Al salts present in the soil e.g. Guam, and the Kii peninsula of Japan. Perl, Gajdusek, Garruto, Yanagihara and Gibbs (1982) were the first to recognise the high incidence of amyotrophic lateral sclerosis and Parkinsonian dementia in such regions. The most striking point inferred from these studies is that under conditions of excess Al exposure, and in contrast to the assumptions outlined above, Al can be absorbed into the body.

Perhaps the most discussed neurotoxic effect associated with Al, is its implication as a possible causative factor in

Alzheimer's disease. Alzheimer's disease is a progressive neurodegenerative disorder which results in the gradual loss of cognitive abilities (Crapper, Krishnan and Dalton, 1973; Coyle, Price and DeLong, 1983; Mann, 1988). It is characterised neuropathologically by the presence of senile plaques (SP) (Kidd, 1964), which consist of a central core of amyloid protein, and neurofibrillary tangles (NFT) which are dense tangles of degenerating nerve terminals and consist of paired-helical filaments (Kruck and McLachlan, 1988; Mann, 1988). Neurochemically, reductions in the enzymatic activities of choline acetyltransferase (ChAT) and acetylcholine esterase (AChE) can be measured, the extent of which varies in different brain regions (Davies and Maloney, 1976; Perry, 1978; Reisine, Yamamura, Bird, Spokes and Enna, 1978; Whitehouse, Price, Struble, Clark, Coyle and DeLong, 1982). Changes related to other neurochemical systems in Alzheimer's disease are not as clearly defined. Davies (1979) found a small reduction in glutamine decarboxylase in 3 out of 20 brain regions investigated and decreases in γ-aminobutyric acid (GABA) receptors have been reported (Reisine, *et al.*, 1978). Increases in dopamine (Wenk and Stemmer, 1981) and noradrenaline (Yates, Ritchie, Simpson, Maloney and Gordon, 1981) have also been reported in Alzheimer's disease patients. However, despite the discrepanies with regard to other neurochemical systems, a considerable number of studies agree that the most extensive and specific neurochemical changes associated with Alzheimer's disease are within the cholinergic system. The reduction in ChAT activity and the morphological changes in Alzheimer's disease are correlated with the extent of impairment of cognitive abilities (Perry,

Tomlinson, Blessed, Bergman, Gibson and Perry, 1978; DeBoni and Crapper McLachlan, 1980).

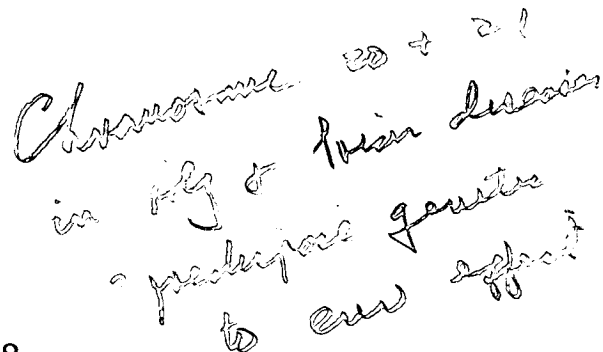
Since these initial findings studies have concentrated on the experimental induction of neurodegeneration similar to that found in the human brain. Crapper, Krishnan and Dalton (1973) induced NFT's following injection of $AlCl_3$ into the ventral hippocampus of the cat. Similarly, tangles have been induced in the brainstem and spinal cord of the rabbit after intracisternal injections of $AlCl_3$ (Yates, Gordon and Wilson, 1976). It is of interest to note that rats do not develop NFT's even when the dose injected is 5 or 6 times greater than that required to induce them in the cat (King, DeBoni and Crapper, 1975). Adult animal models of neurodegeneration have been complicated by the development of other neurologic signs. Rabe, Lee, Shek and Wisniewski (1982) have developed a model in the immature rabbit, by intracranial injection of $AlCl_3$, which induces neurodegeneration and learning deficits but with no other neurological signs.

However, these animal models have been considered limited in their application to the human situation, because the morphology of the tangles induced experimentally are not identical to those found in the Alzheimer's diseased brain (Kruck and McLachlan, 1988). Tangle production may vary between species but other neurochemical changes seem to be consistent, as evidenced by the demonstration of a concurrent loss of ChAT in tissue containing tangles in the rabbit (Yates, Simpson, Russell and Gordon, 1980). The experimental induction of NFT's by the application of Al salts has provided evidence for a role of Al in the aetiology of

Alzheimer's disease. Despite their limitations these models provide a starting point for the development of possible therapies.

The controversy remains whether Al is the primary causative factor in the aetiology of Alzheimer's disease or is secondary to pathological changes resulting from an insult which then cause Al to accumulate within specific susceptible brain regions. Further support for the association of Al with Alzheimer's disease has come from studies which have found Al in the SP and NFT of Alzheimer's diseased brains (Crapper, Krishnan and Dalton, 1973; Perl and Brody, 1980; Candy, Oakley, Klinowski, Carpenter, Perry, Atack, Perry, Blessed, Fairburn, and Edwardson, 1986; Perl and Good, 1988) and from epidemiological evidence which links the levels of Al in water with the incidence of Alzheimer's disease (Martyn, Barker, Osmond, Harris, Edwardson and Lacey, 1989). However, more recently doubt has been cast on the involvement of Al in Alzheimer's disease as some studies, using a different experimental technique, have failed to confirm the presence of Al in SP and NFT of the Alzheimer's diseased brain (Chafi, Hauw, Rancurel, Berry and Galle, 1991; Landesberg, McDonald and Watt, 1992)

The propensity to develop Alzheimer's disease is not the consequence of any single factor but may result from exposure to environmental agents or from genetic susceptibility or an interaction of the two.



1.3.1 Aluminium and Behavioural Teratology

The information reviewed above indicates that, under normal conditions, the body is effective in limiting the entry of Al but when Al levels are high this may not be the case. This points to a need for an increased awareness of the possible toxic role played by Al. There must be concern as the amounts of Al entering the environment are increasing. With the increase in the release of sulphuric and nitric acids into the atmosphere from industry there is a corresponding increase in Al being leached from the soil into surface water (Cronan and Scholfield, 1979; Savory and Wills, 1991). Industry also causes increased levels of Al in the area surrounding Al smelters and there have been accidents with the content of aluminium sulphate ($Al_2(SO_4)_3$ in domestic water supplies, several of which have been reported recently (see for example Cross, 1990). Moreover, Martyn, *et al.*, (1989) have related the level of Al in the drinking water in England and Wales with the incidence of Alzheimer's disease. With this increase in environmental Al there is an increased likelihood of unknowingly being exposed to Al. This is of particular concern with respect to the developing foetus which is, as mentioned in chapter 1, in general more susceptible and is affected at doses far lower than those required to produce changes in the adult CNS (Vorhees and Butcher, 1982; Riley and Vorhees, 1986).

Of greater relevance to behavioural teratology is the accumulating clinical evidence which points to Al loading in infants (see the Introduction to chapter 6 for further details) following exposure to antacids containing high levels of Al (Griswold, Reznik,

Mendoza, Trauner and Alfrey, 1983; Andreoli, Bergstein and Sherrard, 1984), from intravenous feeding of infants (Sedman, Klein, Merritt, Miller, Weber, Gill, Anand and Alfrey, 1985) and from Al present in infant formula (Freundlich, Zilleruelo, Abitol, Strauss, Faugere and Malluche, 1985). Exposure via these routes is of particular concern considering the known immaturity of metabolic pathways (Weaver, Laker and Nelson, 1984) and the blood-brain barrier in infants (Statz and Felgenhauer, 1983).

In the only documented study of accidental exposure of pregnant women to high levels of oral Al, Golding, Rowland, Greenwood and Lunt (1991) did not find a greater incidence of perinatal death, low birth weight, premature delivery or congenital malformations. There was an increased incidence of talipes (club foot) among exposed infants (4 cases compared to 1 control). However, this paper considered reproductive measures only and did not include any behavioural studies.

Detailed studies on the consequences of exposure to Al compounds during pregnancy and the early postnatal period are still lacking. Indeed in a recent review (Savory and Wills, 1991) no reference was made to Al's possible effects on reproduction. In recent years some attention has been given to such effects on the developing foetus but a full evaluation of risk effects has not been undertaken. This is of concern since exposure of the developing nervous system to insults may have very different consequences from those resulting from exposure of the adult nervous system (e.g. Wong and Klassen, 1982).

Proliferation of research within this field may have been hampered by the lack of precise knowledge of the pharmacokinetics

of Al and from the lack of a suitable radioisotope. In addition, early work in this area yielded few adverse effects of exposure (Myers and Mull, 1928; MacKenzie, 1932; and more recently Schroeder and Mitchener, 1975). For example, Myers and Mull (1928) fed albino rats a diet supplemented with 2mg of Al over 100 days. They did not find any differences in litter size or the number of stillborn. Schroeder and Mitchener (1975) exposed Long-Evans rats to 5ppm aluminium potassium sulphate in their drinking water throughout life. After exposure for one year, male rats were slightly heavier than controls and exhibited an increased incidence of tumors. This was not the case for females and no reproductive measures were reported (Schroeder and Mitchener, 1975).

Although the literature is limited, some studies do exist which have considered the effects of exposure to Al compounds during pregnancy and the early postnatal period, and these are reviewed in the introductions to the relevant chapters (see chapters 3 and 6). Within these studies it is difficult to draw conclusions, as different studies have employed different Al salts. Such salts have differential solubilities which can result in altered levels of Al in tissues even when the dose applied initially is similar (LeBlondel and Allain, 1980; Krueger, Morris, Suskind and Widner, 1985; Martin, 1986). As has already been noted, there are species differences in the sensitivity to Al which further complicates the issue.

Of those studies which have addressed this question, most have been concerned with effects on reproduction only and have not followed subjects into adulthood to test whether *in utero* exposure to Al has persistent effects. There are no studies which have measured

any neurochemical changes occurring as a result of gestational exposure even though research has implied an association between Al and the cholinergic system. For this reason an assay for ChAT activity, as a cholinergic system marker, has been included in some of the experiments described in this thesis. Assays were performed on a number of brain regions because, as noted earlier, there are differences in the extent to which ChAT levels are affected in different brain areas and also at different ages.

$Al_2(SO_4)_3$ was used in the experiments reported herein because, unlike other Al compounds e.g. $AlCl_3$ and $Al(OH)_3$, it is soluble in water although only over a limited pH range (pH 3-4.5; Martin, 1986), and because it has been directly implicated in recent environmental accidents.

1.4 EXPERIMENTAL ANIMALS

The house mouse (*Mus musculus*) has several characteristics which have made it the subject of choice in many research laboratories. They are cheap, breed throughout the year, reach reproductive age by 8 weeks, have short gestation lengths (19 ± 2 days) and large litter sizes. They have an adequate repertoire of behaviours which can be measured and quantified. Moreover, a large literature exists which describes such behaviours and their ontogeny thus providing a reference against which any deviation can be assessed. Mice can be tested repeatedly throughout life to monitor the influence of age or developmental stage on the effects of exposure to an external variable.

In addition, and in more specific terms for teratology testing, the choice of rodents as experimental subjects is advantageous as they have the same placentation as primates, the haemochorial, in which the mother's blood system is in very close contact to that of the foetus. This implies that results of drug or chemical testing for teratogenicity in the mouse model are relevant and applicable to the human situation.

Within inbred strains all individuals are virtually isogenic. Hay (1978) puts forward two main advantages of using such strains in the study of behaviour. Firstly, from a statistical point of view, the "error" i.e. the term used to describe the variation between experimental subjects, will be reduced by using individuals whose genetic variation is at a minimum with the result that smaller sample sizes can be used. This has obvious advantages from an ethical viewpoint which aims at reducing the number of animals used in scientific experiments. Secondly, the availability of these strains increases the universality of experiments across laboratories again with obvious advantages. Indeed, Festing (1975) found constancy in the openfield activity of 8 inbred strains of mice tested 13 years apart.

Many groups of researchers have used rodents as subjects for experimental studies for potential behavioural teratogens for the reasons outlined above. Debate exists as to the comparability of results from rodent experiments with the human situation. There is however considerable confidence in the validity of such studies substantiated by extensive comparisons of the effects of several established behavioural teratogens in rodents and humans. Adams (1986) has shown that the particular effect of a given teratogen on

nervous function in mice was essentially the same as those produced in humans exposed prenatally. Chen, Driscoll and Riley (1982), for example, have found that exposure of pregnant Long-Evans hooded rats to ethanol (35% ethanol-derived calories) during gestation days 6-20, resulted in their offspring exhibiting increased latencies to attach to a nipple during days 6-12 postpartum. This suckling deficit within the treated group was not present at 13-21 days of age. This is consistent with clinical reports which have shown that human infants exposed to ethanol prenatally have similar transient alterations in suckling behaviour (Streissguth, Landesman-Dwyer, Martin and Smith, 1980). Similar examples of comparability exist for anticonvulsants (Adams, Vorhees and Middaugh, 1990), drugs of abuse (Hutchings, 1990), lead (Davies, Otto, Weil and Grant, 1990) and methylmercury (Burbacher, Rodier and Weiss, 1990).

Since subtle behavioural and psychological changes are not always immediately apparent, the attribution of such an effect to a substance taken by the mother during pregnancy is difficult. Animal models can provide a starting point for the identification of such causal pathways.

A further problem in identifing and assessing behavioural teratogenicity in humans is genetic variability. Genetically based differences in behaviour and in the rate of development must be taken into consideration when assessing the effects of exposure to teratogens. Again the use of inbred strains provides a clearer picture. As described in 1.2*v*, investigations into the effects of an agent on the developing foetus have revealed large variations in the response of different species to a teratogenic agent and similar variations between strains of the same species. For this reason, two unrelated

strains of mice (CBA/T6 and C57BL/6J), which are known to differ in a variety of ways (van Oortmerssen, 1971; Stewart, Manning and Batty, 1980; Ammassari-Teule and Caprioli, 1985), were used in this study. They form a basic tool for investigating how far any effects of Al can be considered general or are of wide applicability.

The experiments described in this thesis were of longitudinal design; testing of the same subject continued throughout the postnatal period and into adulthood, representing different developmental stages. This enables one to monitor whether effects from exposure are transient and may be recovered from, persist throughout life or whether normal decreases in abilities with age are enhanced. Moreover, it was possible to assess the presence of any delayed behavioural and neurochemical effects resulting from early prenatal exposure which may only arise late in development.

1.5 AIMS

The study aimed at investigating the effects of prenatal exposure to Al on aspects of behavioural development and neurochemistry of two inbred strains of mice. Al was administered via both intraperitoneal and oral routes to assess the contribution of the route of administration on effects resulting from exposure to Al. The protocol includes a wide range of behavioural tests carried out over a wide range of ages. When there were sufficient subjects to enable culling at different ages, ChAT activity was measured at different developmental stages to determine whether effects from exposure to Al are transient and are recoverable, or persist into adulthood. It was also hoped that any changes in behaviour could, at

least to some degree, be correlated with neurochemistry although it is recognised that there is no simple link between brain and behaviour and that a number of neurotransmitter systems may be involved in the expression of a particular behaviour.

Unlike many other studies in behavioural teratology which have only considered one variable, this study aims at providing a broader view of possible effects of prenatal exposure to Al by taking behavioural, neurochemical and genetic aspects into consideration.

CHAPTER 2

2.1 GENERAL MATERIALS AND METHODS

A number of procedures are common to each experiment and these are presented below. Where methods are specific to a particular experiment they are described in the relevant chapter.

2.1.1 Experimental animals

Mice from two inbred strains (CBA/T6 and C57BL/6J, from hereafter referred to as CBA and C57), were used as experimental subjects. These strains have been bred in the animal house of the Zoology Department since 1978. Virgin females between 10-12 weeks of age were mated with males from the same strain. Females were checked daily for vaginal plugs. On identification of a plug they were individually housed in standard plastic cages (31 x 13 x 12cm^3) lined with wood shavings. All mice were maintained in an air-conditioned room with a reversed 12 hour light/dark cycle (white lights on 21.00hr) and at a temperature of $22\pm2^{o}C$. Forced ventilation provided a constant background noise which minimised any disturbance to the mice caused by the experimenter's movements. Food (SDS breeding diet) and tap water with added chlorine were freely available.

2.1.2 Treatment

On gestation day 10 (Gd10), pregnant females were randomly assigned to one of two groups. The allocation of treatments was coded to minimise any observer effect. In each

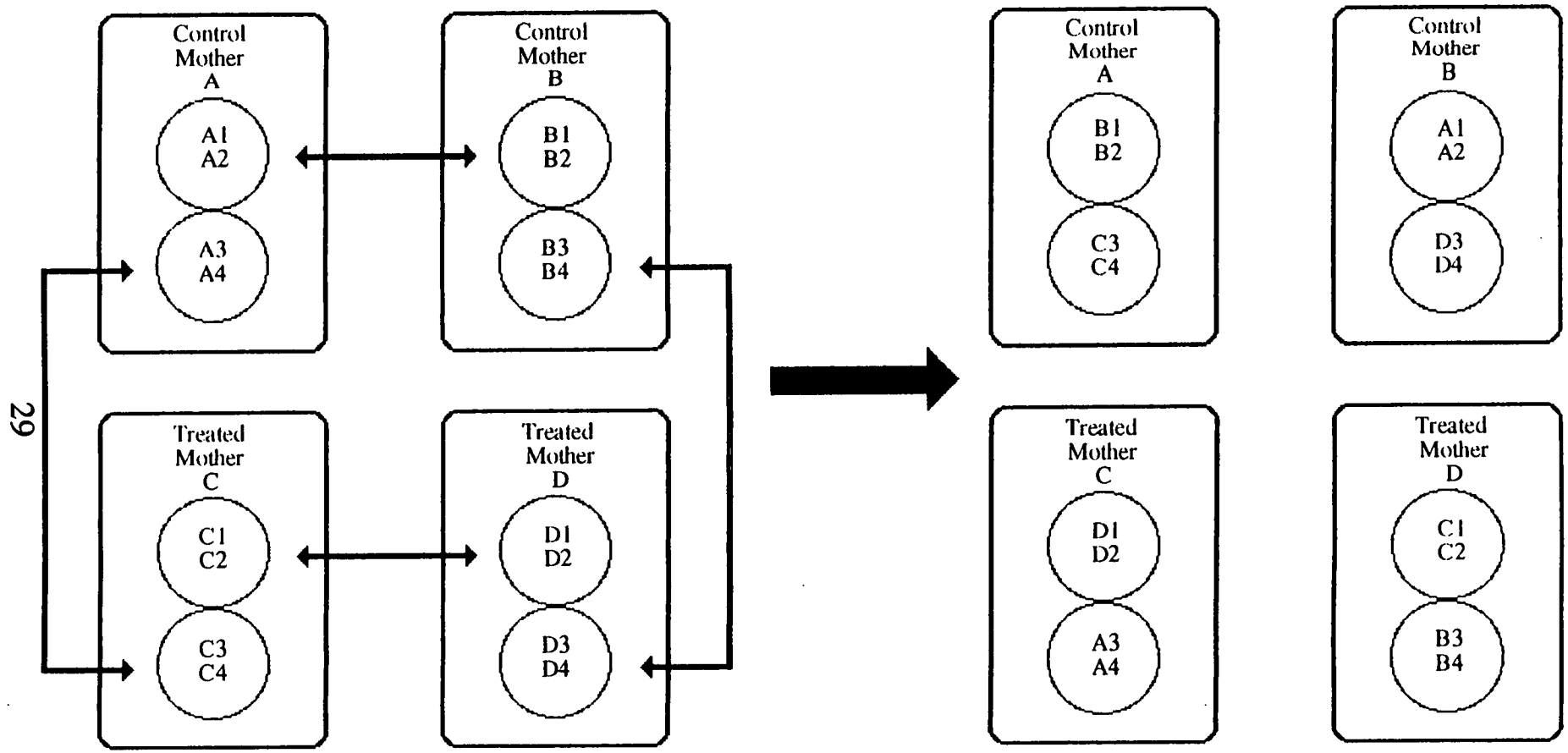
experiment one group was administered $Al_2(SO_4)_3.18H_2O$ either intraperitoneally (i.p.) or orally via the drinking water, at a particular dose and for a specific period of gestation. The exact treatment received is given in each experimental chapter. The control group received the equivalent volume of saline acidified to the same pH as the Al solution (pH=4.1). Maternal weights were recorded during gestation. On Gd18 half of the pregnant females were removed, their foetuses used for tissue culture in an experimental series which does not form part of this report.

2.2 PROCEDURES

The remaining pregnant females were checked twice daily for pups (09.30a.m and 17.00 p.m). On the day of birth (postnatal day 0, Pd0) mothers were allowed to deliver their offspring normally, litter size was recorded and the sex of each pup was determined. On Pd1 all pups born were weighed and each litter was culled to four pups, leaving two males and two females where possible. This litter size was considered sufficient to elicit normal maternal care but small enough to prevent competition between pups which may lead to undernutrition in large litters reared by inbred mothers.

Each pup was toe-clipped for individual identification. Male pups were toe-clipped on digits 1 and 3, and females on digits 2 and 4 of the left paw. The pups were cross-fostered using a balanced split-litter total cross-foster procedure (Chariotti, Alleva and Bignami, 1987), with other litters born within the same 12 hour period (see Fig. 2.1). Thus, each mother received two control and

Fig. 2.1: Diagram illustrating the balanced split-litter total cross-fostering procedure.



1 and 3 = male 2 and 4 = female

two treated pups from other litters and in this way none of them reared any of their own pups. Hence the pups could be divided into four experimental groups; control pups cross-fostered to control mothers (**Cc**), control pups fostered to treated mothers (**Ct**), treated pups fostered to control mothers (**Tc**) and treated pups fostered to treated mothers (**Tt**).

2.2.1 Pup behaviours

Each pup underwent two types of behavioural testing, which were carried out on every third day from Pd3 to Pd18, during the early part of the dark phase when pups are considered to be at their most active (Alleva, Aloe and Laviola, 1986). Firstly, the pup was placed in the centre of an openfield measuring 40 x 40cm^2, the base of which was divided into 10cm^3 squares. Uniform lighting was achieved by a 25W bulb suspended above and in the centre of the field. Observation was facilitated by using a mirror fixed above the openfield which obscured the experimenter from view by the pups. The number of squares crossed and several items of behaviour were observed for three minutes and recorded on a keyboard linked to a computer. Squares were defined as crossed when two paws had crossed a line. The pup behaviours recorded were as follows :-

Pivoting - The pup is attempting to crawl but due to the immaturity of its hindlimbs only manages a circular movement.

Crawling - The pup is actively moving around the openfield.

Rearing - The pup is standing on its hindlimbs.

Edgeon - The pup is rearing against a wall of the openfield.

Grooming - The pup is licking any part of its body.

Still - The pup is still and not involved in any of the other behaviours.

Headup - The pup is still except for raising its head.

Onback - During one of the other pup behaviours, the pup falls over onto its back.

In addition, the pups underwent a modified version of the Fox battery of tests for sensory-motor development (Fox, 1965). The following tests were carried out :-

Righting reflex - Pups were placed on their backs and the ability to reorientate themselves onto all four paws was scored. By Pd9 slow righting gives way to swift righting involving one continuous and co-ordinated movement. The change is unmistakable and for swift righting a measure of time to complete this movement was measured.

Cliff aversion - Pups were placed with forepaws over the edge of a wooden block, the occurrence of a turning response away from the edge was recorded.

Forelimb grasping - The underside of a fore paw is gently stroked with a cocktail stick and the strength of flexion of the digits is scored.

Pole grasping - The stick is placed under the forelimbs and slowly raised to measure the ability of the pup to hold on to the "pole". In a mature response the pup clambers up using its hind feet.

Screen climbing - A pup is placed at the bottom of a horizontal wire mesh which is then moved to the vertical. The ability of the pup to hold on to the screen and climb to the top was measured.

Eye opening - An adult response was recorded when both eyes were fully opened presenting a full circular appearance.

This order of testing remained unchanged throughout the experiment and each pup was tested once per occasion. The pups were assessed on a scale from 0 to 3, 3 being the equivalent of a mature response. Finally, the pups were weighed and returned to the home cage. Individual litters were tested at a different time during each experimental day to counteract any circadian effects. Handling of the pups was kept constant and to a minimum throughout testing to prevent the enhancing effect this is known to have on pup development (Levine, 1958).

2.2.2 Tests at weaning

On Pd21 each pup was observed in the openfield, this time for five minutes, again recording the number of squares crossed using a hand tally. At the end of this period, a novel object in the form of a white cube (65 x 65cm^3), was introduced into the centre of the arena once the subject had moved into one of the corner squares. Latency to contact the object was recorded, each subject being given a maximum of three minutes to do so, after which the test was terminated. Timing was stopped when the subject made actual contact with the novel object, often with the tip of the nose first. The pups were then weaned into single sex cages in groups of 2-3. Weekly weights were recorded throughout the duration of testing.

2.2.3 Maze testing

Ten week old male mice were tested in an 8-arm radial maze. Females were not used because it is known that males are superior in learning tasks of this kind (Schulze, 1976; Lamberty and Gower, 1988). This may be due to the possible effects of the oestrous cycle on performance in the maze (Tsujii and Hoshishima, 1979) or because females may be less able to withstand the weight loss associated with food deprivation. The maze consisted of a circular base into which the arms were slotted. This formed an octagonal central platform (diam 100cm) from which eight arms (length 40cm, width 8cm) radiated. The walls of the arms were constructed from black plexiglass. Each arm was also covered with

clear plexiglass to prevent subjects from escaping or from passing from one arm to another without returning to the centre. Each arm was numbered to enable recording of arm entries. A food dish was placed at the end of each arm and was baited with a small piece of food pellet prior to each trial. A lamp (15W) 30cm above the central platform illuminated the maze. The maze was positioned on a table and its surroundings were rich in a variety of visual cues.

2.2.3a Food deprivation

Five days before the beginning of the experiment subjects were maintained on a restricted feeding schedule in which food was limited to 2 hr. out of every 24 hr period. By the start of testing this schedule had reduced their body weight by 10-15%. Weights were measured daily to ensure that weight loss was consistent between groups. If this was not the case the feeding schedule was altered accordingly. Subjects had unlimited access to water in their home cages throughout the experiment.

2.2.3b Maze procedure

The procedure was based on that originally used by Olton and Samuelson (1976). Mice were food deprived for three days prior to testing and on the day before testing (day 3) each subject was individually introduced into the apparatus for 10 minutes habituation, each arm having been previously baited. The experiment commenced on day 4 when each subject was tested in turn, once a day, until the majority of subjects had learnt the maze. The subject

was confined to the central platform by means of a cardboard container. After 90 seconds had elapsed the container was removed and the subject was able to freely explore the apparatus. The sequence of arm entries was recorded until the subject had entered either (a) all 8 arms once, (b) made 16 entries into arms or (c) until 10 mins had elapsed, which ever condition was met first. An arm choice was considered to have occurred when the subject had run at least half way down an arm. An error was scored if the subject revisited an arm, failed to visit one of the arms or successfully entered a baited arm but did not eat. The overall criterion for completion of the test was based on that employed by Pick and Yanai (1983). A subject was judged to have reached criterion when it had entered all 8 arms on the first 8 visits on two consecutive days. This provided information on the number of correct entries made during the first 8 attempts, the number of visits required to enter all 8 arms once, and the number of days to reach criterion. I considered that this criterion would be a more reliable one for learning ability than a perfect score on one day only which could result from chance. The maze was cleaned between trials to remove faecal boli, urine and odour cues, and washed with disinfectant at the end of each test day. Each subject was eliminated from the experiment when it had reached criterion. At the end of the experimental period all subjects were returned to their normal feeding regime.

2.2.4 Activity testing at maturity

Twenty-two week old female mice again underwent testing in the openfield. Each mouse was confined in a holding box for 60

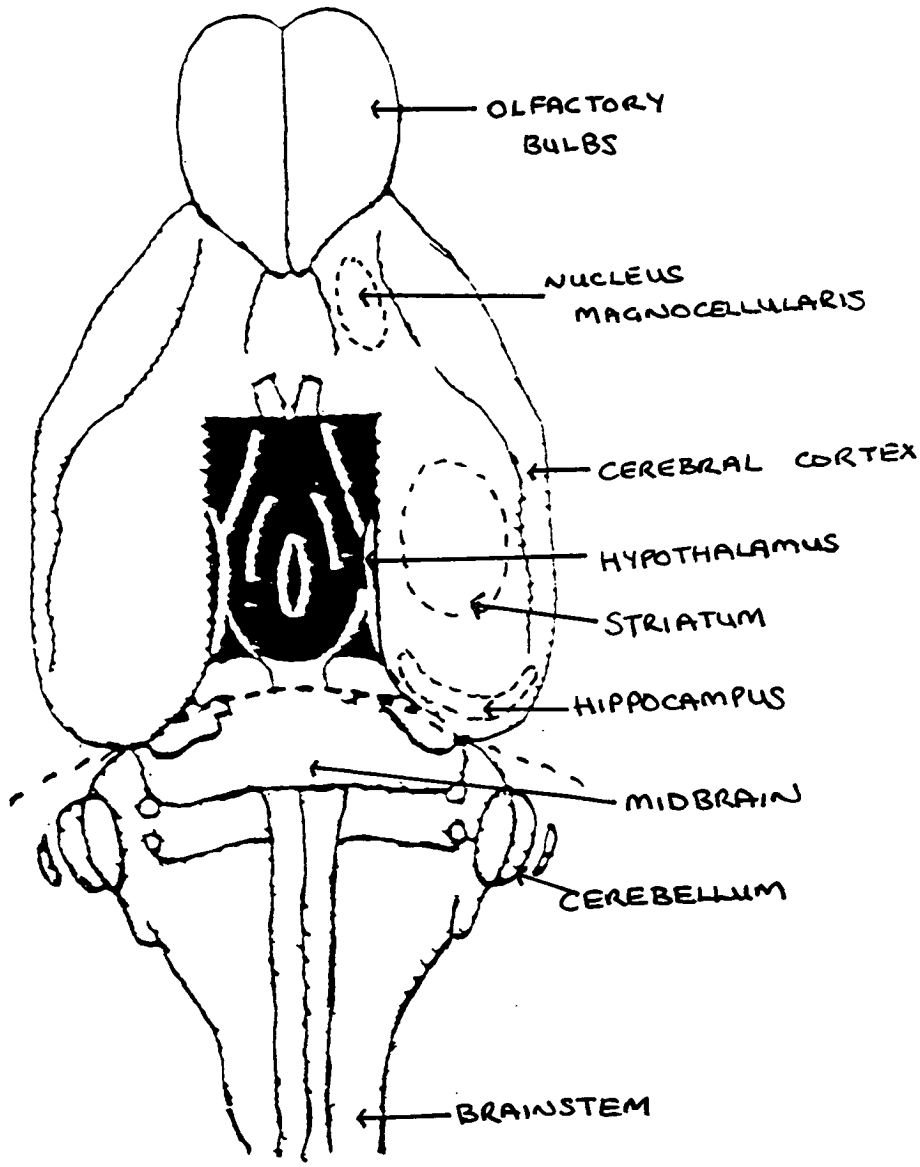
seconds after which a side door was lifted and latency to enter the openfield was recorded. The side door was closed after the animal had entered the openfield to prevent reentry into the holding box. The number of squares crossed per minute was noted over a five minute period on three consecutive days. On day 4 the same novel object as was used in the weaning tests was introduced into the centre of the arena at the start of the test. The subject was again placed in a holding box and released into the openfield, this time the latency to contact the object was recorded in addition to the latency to enter the openfield and the number of squares crossed.

2.2.5 Assay for choline acetyltransferase (ChAT) activity.

Animals were decapitated, the olfactory bulbs removed and the brains were dissected into eight regions - the brainstem, cerebellum, midbrain, hypothalamus, nucleus magnocellularis, hippocampus, striatum and cerebral cortex, using the diagram in Glowinski and Iversen (1965) as a reference (see Fig. 2.2). Each dissected portion was placed into labelled cuvettes and stored at -70^{o}C until assay. Tissue samples used for the assays represent pooled brains from 3-4 individuals not divided by sex.

ChAT activity was assayed in each of these brain regions by the method of Glover and Green (1972). Tissue samples were homogenised in saline containing butan-1-ol. Separate 50μl samples of the homogenate were pipetted into 3 test-tubes followed by 50μl of the assay mix which contained 140M ^{14}C acetyl CoA, 10mM choline, 200mM potassium chloride, 20mM phosphate buffer at pH 7, 0.2mM EDTA and 0.2mM eserine sulphate, all of which are

Fig. 2.2



TAKEN FROM GLOWINSKI AND IVERSEN (1965).

essential for the reaction. This was then incubated at 37°C for 20 minutes. The ^{14}CoA formed according to the equation below (Cooper, Bloom and Roth, 1981), was extracted with potassium mercuric iodide in octane. Aliquots of this were placed in scintillation tubes, scintillation fluid added and counted in a Packard Tri-Carb 460 scintillation counter at 80% efficiency.

Acetyl CoA + choline ---------> acetylcholine + CoA

The protein content of the brain homogenates was determined by the Biorad assay developed by Bradford (1976), to provide the standard against which activity could be related. 5 µl of each homogenate was placed in a cuvette to which 2.5mls of the Biorad solution was added. The standard was bovine serum albumin at 1mg/ml. Absorbance was read at 595nm. The activity of each tissue sample is expressed in pmoles per minute per mg protein.

2.3 STATISTICS

Pup behavioural data was analysed using a programme, "Keytime", which provides information on the frequency and duration of each press made by the observer on the computer keyboard (Deag, 1983b). The normality of the data was analysed using the Nscores option in the Minitab statistical package and from histograms of the distributions (Ryan, Joiner and Ryan, 1985). Normally distributed data was analysed using the P2V programme for analysis of variance (ANOVA) on the B.M.D.P statistical package (Dixon, 1988) with prenatal treatment, foster mother

treatment and sex as the dependent variables (all fixed effects, see Snedecor and Cochran, 1988 p322) and either postnatal day or minute of test as the repeated measures factor, using the Greenhouse-Geisser correction. In experiments where a fostering procedure is not used, a dependent variable should be included to control for litter effects. As the design of the experiments described in this thesis incorporated a comprehensive fostering procedure, this was not felt to be necessary.

Nonparametric tests in the form of the Kruskal-Wallis one-way ANOVA were used when the data did not approximate to normality, using the B.M.D.P. 3S programme which includes a multiple comparisons option (Dixon, 1988). The total weight gained during gestation and birth weights were compared using Student's *t*-test. Chi-square tests were used to analyse the categorical data generated by the Fox tests (Ryan, Joiner and Ryan, 1985).

The neurochemical data presented in chapter 3 was analysed using Student's *t*-test and that described in chapter 6 by ANOVA. Significance was taken to be at the $p<0.05$ level and unless stated otherwise, the mean and standard error of data sets are given.

CHAPTER 3

Effect of gestational exposure (Gd10-13) of CBA mice to injected aluminium sulphate.

3.1 INTRODUCTION

As discussed in chapter 1, studies exposing adult animals to Al, in an attempt to elucidate the mechanisms by which it exerts its neurotoxicity, are in abundance. In contrast, research into Al's potential as a physical or behavioural teratogen are rare. With the ever increasing amounts of absorbable Al entering our modern environment, it is surprising that more work on gestational exposure to Al has not been undertaken.

Administering a substance by injection enables one to discharge a given amount accurately in relation to the body weight of the individual. In this chapter I describe experiments in which pregnant CBA mice were exposed to 200mg/kg body weight of aluminium sulphate ($Al_2(SO_4)_3$) by repeated intraperitoneal injections during gestation days 10 to 13. The aims were to assess both the immediate and long-term effects of Al on the physical and behavioural development of the offspring.

3.1.1 Exposure to aluminium compounds by injection

It is important to note those studies we do know which have considered the effects of exposing the developing foetus to Al via injection.

Acute intraperitoneal (i.p.) administration of $AlCl_3$ at a dose of 40mg/kg body weight on gestation day 9 or 13 only, to

female albino rats, did not produce any overt signs of toxicity to mothers or their offspring (Benett, Persaud and Moore, 1975). Conversely, daily i.p. injections of $AlCl_3$, at varying doses (75, 100 or 200mg/kg body weight) to pregnant rats from days 9-13 or 14-18 of gestation resulted in a higher occurrence of maternal and foetal deaths and increased foetal resorptions at the highest doses, irrespective of the period during which treatment was administered. The mean body weight of the mothers and their offspring, and the pup's crown-rump length, were reduced by the 75 and 200mg/kg doses of $AlCl_3$ between Gd9-13. Skeletal defects were greater in offspring of mothers treated at a dose of 100mg/kg during both periods of gestation when compared to control pups (Benett, Persaud and Moore, 1975).

A further study involving exposure to $AlCl_3$ via i.p. injections at different doses (ranging from 100 to 300mg/kg body weight) during gestation days 7 to 16, increased the Al concentration in the maternal liver, placenta and in the foetuses of BALB/c mice. This led to an increase in foetal resorptions and reduced foetal weight even in the absence of any obvious toxic effects to the mother (Cranmer, Wilkins, Cannon and Smith, 1986).

Yokel has carried out extensive work on the effects of systemic exposure to aluminium lactate (AlLact) on rabbits to investigate its toxicity during different developmental stages. Subcutaneous (s.c) administration of AlLact to lactating does during days 4-29 postpartum at different concentrations (0, 25, 100, 400 or 800 μmol/Al/kg), resulted in maternal weight loss and death in the highest dose group (Yokel, 1985). Interestingly, there was a biphasic effect of exposure to Al on weight gain of both does and their

suckling offspring; those exposed to 25 or 100 μmol/Al/kg had a greater weight gain compared to controls, whereas those given AlLact at the higher doses gained less weight than controls. However, the tissue concentration of Al in the does did increase with dosage. Milk production, as measured by a decrease in offspring milk consumption, was lower in treated does. Yokel (1984) concluded that there was little toxic effect to suckling offspring from exposure of their lactating mothers to AlLact because of the estimated low levels of Al to which the offspring were actually exposed. Exposure to the highest dose (800 μmol/Al/kg) proved to be lethal.

Gestational exposure of does to AlLact at a dose of 400 μmol Al/kg/inj between days 2-27 of pregnancy resulted in a significant increase in stillbirths and postnatal mortality (Yokel, 1985). The biphasic effect of Al exposure on weight gain was also present after gestational exposure. Moreover, *in utero* exposure to low doses of Al enhanced performance in a conditioned reflex learning test compared to an attenuated response when Al was administered at high doses. Tissue concentration of Al was correlated with the dose of Al to which the doe was exposed, but the effect on milk production was to lower it with increasing dose (Yokel, 1985). It is of interest to note that this is the only study which included a cross-fostering procedure, involving the rearing of 3 fostered and 3 biological offspring per litter. It was not only the natural offspring of mothers exposed to 400 μmol/Al/kg which gained less weight but also control offspring fostered to them.

In summary, it is clear that most studies of gestational exposure to Al have limited themselves to measuring the immediate

toxic effects in terms of maternal growth rates and foetal weights. Yokel (1985) provides the only example of a longitudinal study of changes to exposed offspring with time. The work presented below aims to investigate any possible latent or continual effects of *in utero* exposure to Al.

Some of the work described in this chapter has been published (Clayton, Sedowofia, Rankin and Manning (1992), see Appendix A).

3.2 EXPERIMENT 1

3.2.1 Treatment

Batches of females exposed to males and found to be carrying a vaginal plug were randomly divided into two groups. One group was injected i.p. with 200mg/kg aluminium sulphate ($Al_2(SO_4)_3$) daily from Gd10 to Gd13 inclusive, whilst the control group received injections of the equivalent volume of saline acidified to the same pH (pH=4.1-4.3) as the Al solution with sulphuric acid. It had been intended to administer the Al from Gd10 to 17, but obvious hardening of the skin around the injection site led to the decision to stop injections after four days only.

3.3 GENERAL PROCEDURES

The sequence of testing and the methods described in section **2.2** were employed in this experiment. Briefly, all pups were weighed on Pd1 and cross-fostered with litters of the same age. Every third day from Pd3 to Pd18 pups were weighed, underwent tests for sensory-motor development and were observed in an openfield for 3 minutes. At weaning the number of squares crossed in the openfield were recorded and a novel object was introduced into the centre square at the end of the test.

3.3.1 Maze testing

Following completion of the 8-arm radial maze test (see *2.2.3b*) subjects were rested for 5 weeks and then retested in the maze, to investigate the effects of prenatal exposure to $Al_2(SO_4)_3$ on learning and recall. They were food deprived for the same length of time but were not given a pre-test day for habituation. The procedure remained the same for the rest of the session. On completion of the second maze, half the control and treated subjects were sacrificed for neurotransmitter enzyme assay (see *2.2.5*).

Adult activity tests were carried out at 22 weeks of age and weekly adult weights were taken from weaning until this age.

3.4 RESULTS

The main ANOVA tables and median + lower and upper interquartile ranges are given in Appendix B.

3.4.1 Estimated level of aluminium exposure

With an average weight of 25g, pregnant females injected i.p. with $Al_2(SO_4)_3$ received 0.41mg of elemental Al per day, a total of 1.64mg over the four days of treatment.

3.4.2 Breeding performance

Prenatal exposure of pregnant female CBA mice to $Al_2(SO_4)_3$, at a dose of 200mg/kg body weight between gestation

days 10-13, had no significant effect upon breeding performance. The length of gestation was similar in control and treated groups; the mean time between detection of the vaginal plug and the day of birth was 20.73(±0.3) days for control females (n=11) and 20.38(±0.42) days for the treated group (n=8). The mean litter size for control and treated females was 5.27(±0.65) and 5.57(±0.72) respectively. The sex ratio within these litters was also unaffected, and external examination of the pups did not reveal any morphological differences. Interestingly, in both groups, there was some mortality at birth from females who had a gestation length of less than 20 days. After cross-fostering, 3 dams were eliminated from the experiment (2 control and 1 treated), as they had neglected or killed their fostered offspring. This left a total of 6 control and 7 treated dams, all except one had 4 pups, and no further postnatal mortality occurred. The number of pups in each experimental group was as follows:- **Cc** n=13, **Ct** n=14, **Tt** n=12 and **Tc** n=11.

3.4.3 Maternal weight

Analysis of variance (ANOVA) revealed a significant overall main effect of treatment on maternal weight during gestation ($F(1,11)=5.05$ $p<0.05$). As expected there was no difference in maternal body weight on Gd10. However, thereafter the mean body weight of the treated mothers (n=7) was reduced by 7.7% compared to that of the controls (n=6) by Gd12 (control $\bar{x}=24.12(\pm0.45)$g, treated $\bar{x}=22.27(\pm0.37)$g. Once treatment had ended, the treated dam weight recovered to a mean of 27.2g on day 18. Control mothers did not exhibit a corresponding reduction in weight during the treatment

period but continued to gain weight reaching a mean weight of 30.2g on day 18, 9.8% heavier than treated dams. The mean total overall gain in weight between Gd10 and Gd18 was 7.02(±1.23)g for controls and 4.43(±1.04)g for treated mothers. This difference was not significant.

3.4.4 Pup weight

Mean birth weight was significantly lower among Al-exposed offspring than controls between all pups born (control n=52 $\bar{x}$=1.29(±0.02)g, treated n=38 $\bar{x}$=1.21(±0.02)g; $t(78)= 2.70$ $p<0.009$). This effect held when only those pups which acted as experimental subjects were compared (control n=27 $\bar{x}$=1.33(±0.03)g, treated n=25 $\bar{x}$=1.22(±0.03)g; $t(47)= 2.63$ $p<0.02$). Treated pups weighed 6% less than control offspring. This implies that those pups which were randomly selected for behavioural testing were indeed representative of the population. Analysis was also carried out which considered the treatment the pup's future foster mother received during pregnancy to ensure that the fostering protocol distributed the pups evenly between control and Al-treated mothers. There was no such effect of foster mother treatment on birth weights. Thus, those pups who were of low birth weight to begin with, were not all being allocated to mothers who had received $Al_2(SO_4)_3$ during gestation which might further hinder their development.

Fig. 3.1 summarises the mean body weights during early development for male and female offspring of the four experimental groups. Male and female offspring weights were combined for analysis as there was no significant difference between the sexes.

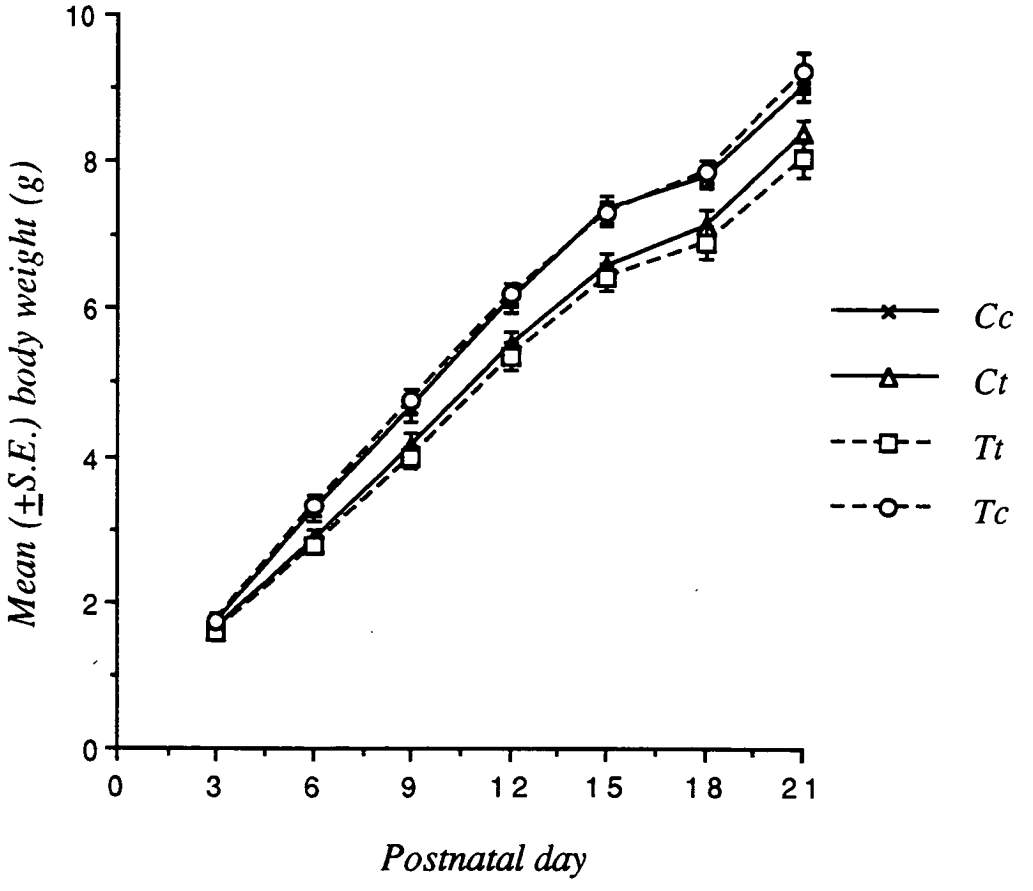


Fig. 3.1: Effect of prenatal exposure to aluminium suphate (200mg/kg, Gd10-13) on CBA pup body weight during the preweaning period.

One treated pup fostered to a treated mother (**Tt**) was removed from the analysis as I considered it to be a runt. There was no significant overall main effect of prenatal treatment on pup weights. However, the treatment groups began to diverge in body weight on Pd6 and this persisted until weaning. From the graph it can be seen that treated pups reared by control mothers (**Tc**) were the heaviest and **Tt** pups the lightest throughout the preweaning period. By weaning **Tc** pups were 13% heavier than **Tt** pups. Thus, the lower birth weight of the treated pups persisted only for treated pups reared by treated mothers. Comparing the results of the **Tc** pups with those of the control pups fostered to control mothers (**Cc**) does not suggest a profound effect of direct exposure to $Al_2(SO_4)_3$ *in utero*. This was also the case when control pups reared by treated mothers (**Ct**) were compared with those of the **Tt** group. Considering the extreme groups, **Cc** with **Tt**, or **Ct** with **Tc**, gives an indication of the combined influence of prenatal and postnatal factors. There was no significant prenatal treatment by foster mother treatment interaction. **Cc** pups were consistently heavier than **Tt** pups, the greatest difference occurring on Pd9 when **Cc** pups weighed 14% more. Likewise **Tc** pups weighed consistently more than **Ct** pups.

Despite the lack of a direct effect of prenatal exposure to $Al_2(SO_4)_3$, the graph clearly illustrates that there is a profound maternal treatment effect on pup body weight which persisted until weaning ($F(1,42)=15.28$ $p=0.0003$), the significant interactive effect of foster mother treatment and day shows that the extent of this effect varied with time ($F(6,252)=14.58$ $p<0.0001$). **Cc** pups and **Tc** pups are grouped in the same part of the graph as are **Ct** and **Tt** pups, i.e. by foster mother treatment. Thus, fostering a treated pup to

a control mother seems to enhance development, by compensating for the effect of an initial low birth weight due to exposure to $Al_2(SO_4)_3$ *in utero*. Alternatively, fostering a control pup to a treated mother adversely affects the physical development of the pup, illustrating that differences must exist in some aspect, or aspects, of the postnatal mother-infant interaction. It is not clear at this time exactly how this effect is produced.

3.4.5 Adult weight

From weaning onwards significant differences in weight between the sexes began to emerge and thus male and female weights were analysed separately. Male weights were not analysed during the periods of starvation prior to maze testing, although weights were recorded during this intervening period to ensure weight loss was consistent between the groups. **Cc** males were consistently heavier than the other groups which had not been the case up to weaning and **Tt** males were lighter. There was no overall main effect of prenatal or foster mother treatment, although there was a trend towards lower weights in mice fostered to treated mothers.

Female offspring were weighed at weekly intervals throughout the experiment from weaning onwards. The trend which existed up to weaning, treated pups fostered to control mothers weighing the most and treated pups fostered to treated mothers the least, was present only up to 6 weeks of age after which the **Cc** and **Tc** females had comparable body weights. **Tt** females continued to weigh less than the other treatment groups (see Fig. 3.2). The

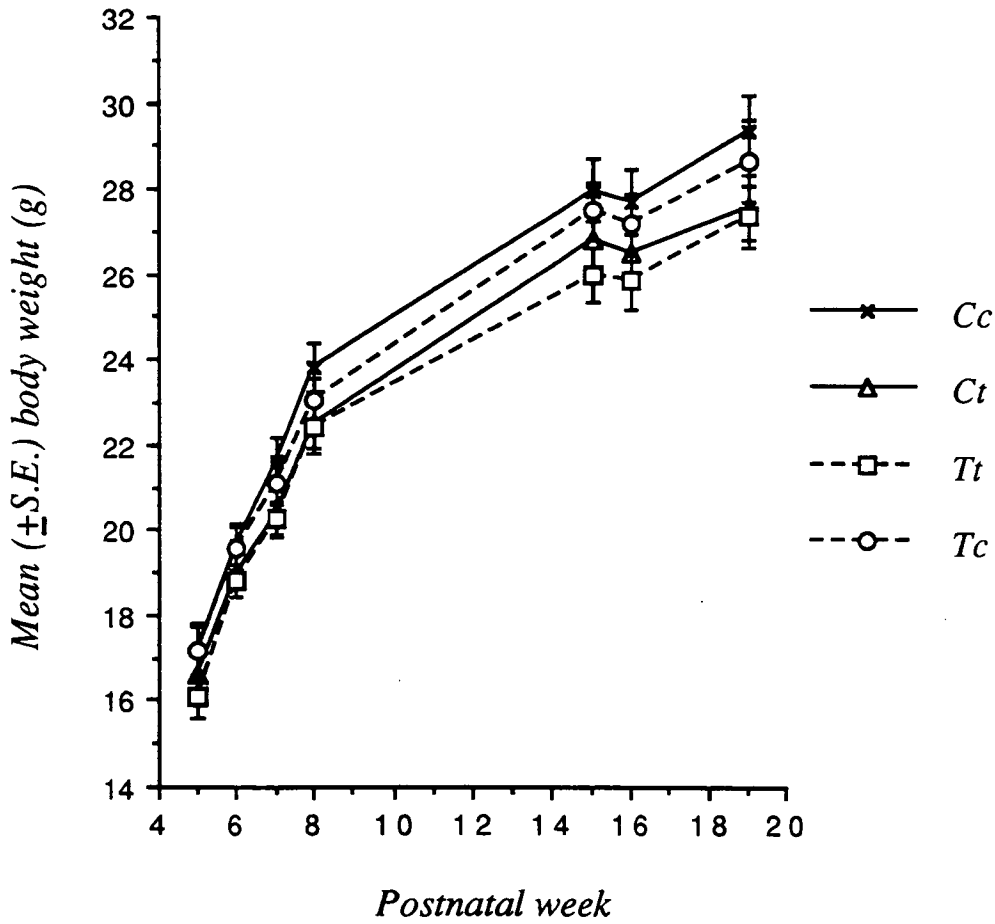


Fig. 3.2: Effect of prenatal exposure to aluminium sulphate (200mg/kg, Gd10-13) on CBA adult female body weight.

significant effect of foster mother treatment present from Pd6, persisted into adulthood in the case of female offspring ($F(1,18)=9.52$ $p<0.007$).

Thus both male and female **Tt** pups continued to be lower in weight than the other groups throughout life with this effect being more pronounced among female mice. It would seem that exposure to $Al_2(SO_4)_3$ may be sex-specific in its effects, being more toxic to females.

3.4.6 Fox tests

The effects on growth noted above were accompanied by delays in the maturation of some of the behavioural components measured by the Fox tests. There was a highly significant difference between the groups for both sexes in the attainment of a mature response for *forelimb grasping* ($X^2(3)=11.901$ $0.01>p>0.001$) and *pole grasping* ($X^2(3)=24.674$ $p<0.001$), both on Pd15 (see Figs. 3.3a and b). With regard to *forelimb grasping*, most of the pups had reached an adult response by Pd15 except for some pups in the **Tt** group. A maternal effect can be seen in the results for *pole grasping* as few **Ct** or **Tt** pups had reached a mature response by Pd15. Similarly, a significant difference between the groups was found for *cliff aversion* on Pd12 ($X^2(3)=10.669$ $0.02>p>0.01$) and *screen climbing* on Pd18 ($X^2(3)=8.069$ $0.05>p>0.02$), again fewer pups in the **Ct** and **Tt** groups had attained a mature response.

Scores on the Fox tests which revealed significant group differences were correlated with body weights on the same day. **Tt**

Forelimb grasping (Fig. 3.3a)

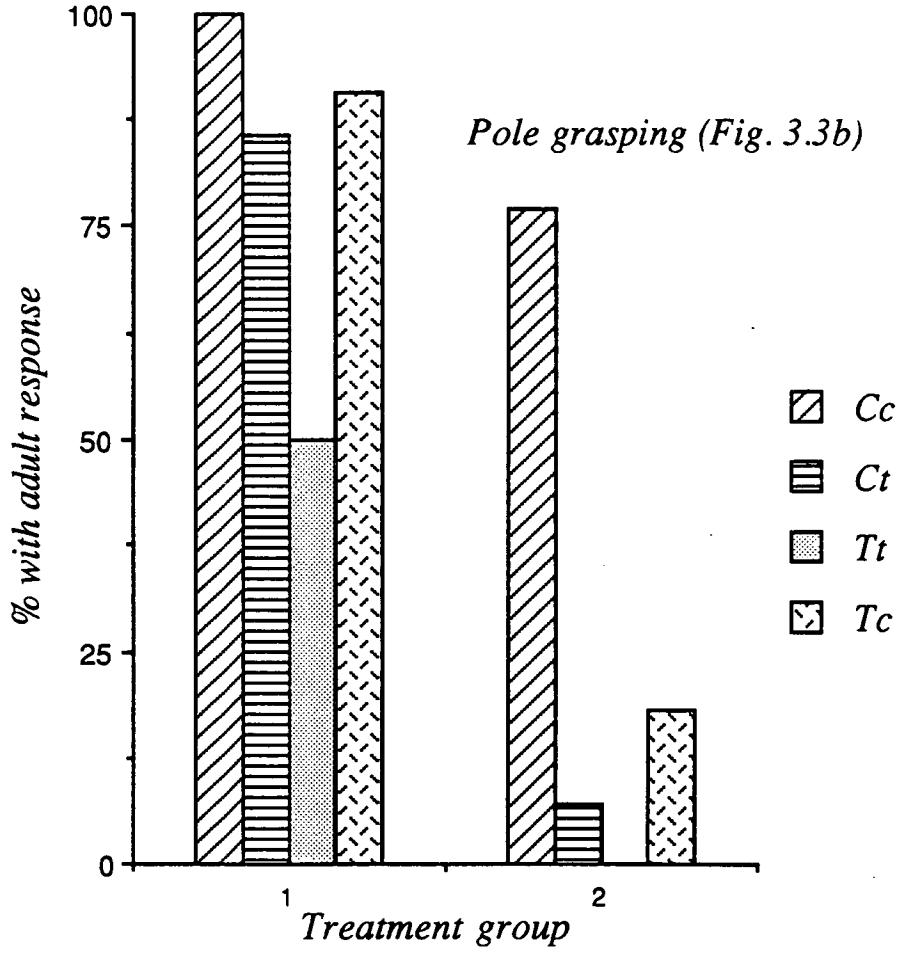


Fig. 3.3: Percentage of CBA pups with an adult response by Pd15.

pup scores were correlated with weight for *slow righting* (**Tt** n=12, r=0.869 $p<0.001$) but this was the only significant correlation.

3.4.7 Pup behaviours

Pup behaviours were analysed from the day on which the particular behaviour was first expressed. Thus, for *crawling*, the behaviour was analysed from postnatal day 6-18, but for *rearing* analysis was from day 12 to 18. This accounts for the difference in the value for degrees of freedom.

Activity - The effects of prenatal exposure to $Al_2(SO_4)_3$ on the ontogeny of pup locomotor *activity* during postpartum days 9-18 is shown in Fig. 3.4. There was a marginally significant effect of foster mother treatment on pup activity; pups reared by treated mothers showed a trend towards diminished *activity* levels compared with controls. There was a highly significant interactive effect of day, prenatal treatment, foster mother treatment and sex ($F(3,126)=4.58$ $p<0.02$). As expected all pups became progressively more *active* with age as confirmed by the significant effect of day of testing ($F(3,126)=93.94$ $p<0.0001$).

Righting - Self *righting* behaviour was evident from Pd3 and successful righting was achieved by Pd9, reflected in the diminished frequency and time spent involved in this behaviour. There were no significant group differences in the frequency or duration of bouts of *righting*.

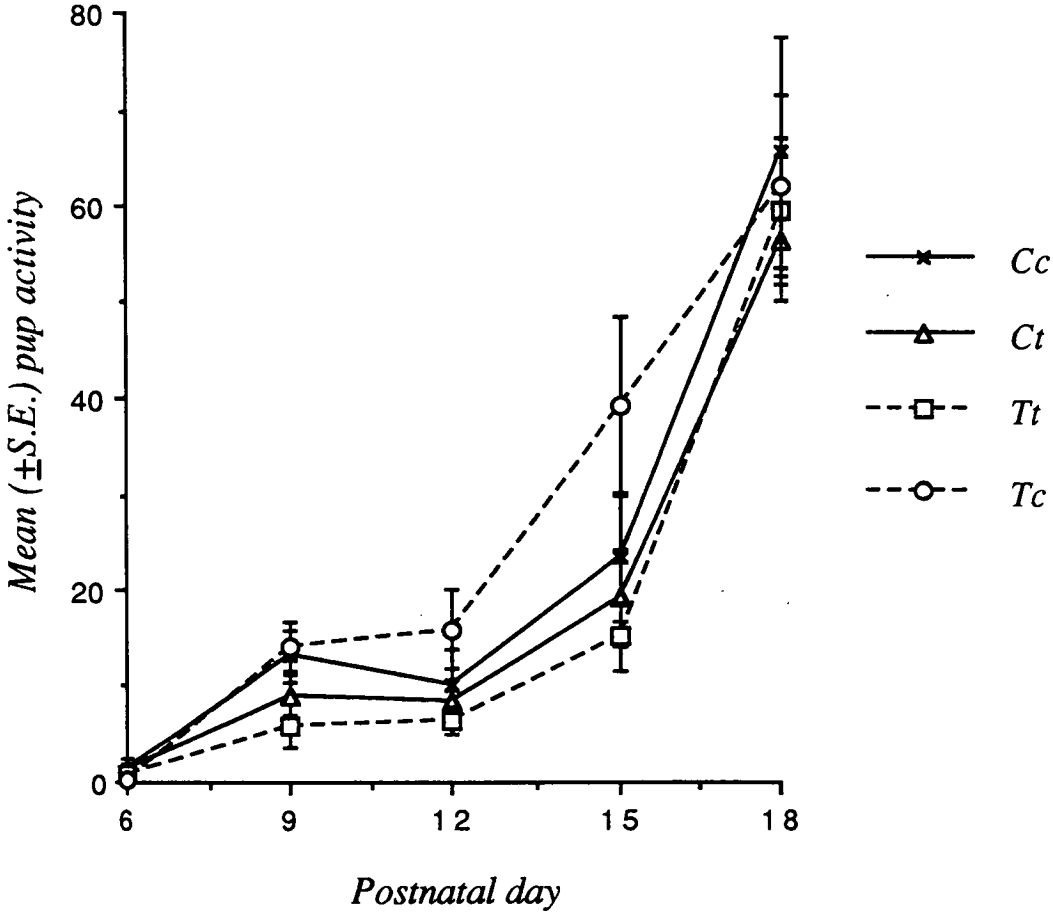


Fig. 3.4: Effect of prenatal exposure to aluminium sulphate (200mg/kg, Gd10-13) on the number of squares crossed by CBA pups in a three minute openfield test.

Pivoting - There were no significant differences in the frequency or duration of episodes of *pivoting* which decreased gradually from Pd3 to 12 for all pups, being replaced by crawling.

Crawling - Pups from all four experimental groups commenced *crawling* on Pd6, although bouts of *crawling* at this stage were usually preceded by episodes of *pivoting*. From Pd9 onwards episodes of *crawling* increased in frequency and duration with age. There was no significant difference between experimental groups in the frequency of bouts of *crawling*, however there was a trend towards a reduced frequency by pups reared by treated mothers. Interactive effects on the duration of *crawling* were present. Day (D), prenatal treatment (PT) and sex (S) interacted to give a significant effect ($F(4,168)=3.73$ $p<0.009$), as did D, PT, foster mother treatment (FMT) and S ($F(4,168)=2.79$ $p<0.04$). Control pups spent more of their time involved in *crawling* compared to treated pups. Pups fostered to control mothers exhibited bouts of *crawling* with longer duration than those raised by treated mothers.

Rearing - The ability to *rear* on hindlimbs was present from Pd9 although some pups would *rear* onto the wall and remain still. The frequency and duration of bouts of *rearing* varied with postnatal day, pups becoming progressively more involved in this behaviour with time. There was a highly interactive effect of all variables (D x PT x FMT x S) on the frequency of *rearing* ($F(2,84)=4.92$ $p<0.02$).

Grooming - Pups became involved in self *grooming* from Pd6 although at this age it usually resulted in them falling over. By Pd12

full body grooming had been achieved and this behaviour increased with age. There were no significant group differences in the frequency or duration of *grooming* bouts. The frequency of *grooming* bouts during the later postnatal days remained low but were of longer duration. Pups fostered to control mothers had episodes of *grooming* with the longest duration.

Headup - This behaviour was present from Pd3 and occurred throughout the preweaning period. There were no significant group differences in the duration of time spent or the frequency of *headup*.

Still - There were no significant group differences in the duration of bouts of being *still*. There was a significant effect of foster mother treatment on the frequency of *still* bouts ($F(1,42)=4.41$ $p<0.05$); pups reared by treated mothers exhibited greater numbers of bouts of being *still*.

Edgeon - Rearing against the walls of the openfield was evident from Pd9 and its occurrence increased with age. There were significant group differences in the frequency ($H(3)=10.22$ $p<0.02$) and duration of time ($H(3)=8.34$ $p<0.04$) involved in bouts of *edgeon* behaviour on Pd12; pups reared by treated mothers were less involved in this behaviour. Group differences were marginally significant on Pd15.

3.4.8 Activity at weaning

Activity scores at weaning were examined by ANOVA with minute of test as the repeated measure. There was no effect of

prenatal or foster mother treatment on activity scores at weaning but they did vary with sex ($F(1,42)=4.17$ $p<0.05$), female offspring being more active than males, and across minute of test ($F(4,168)=3.46$ $p<0.02$). In addition there was a significant interaction between prenatal treatment and sex ($F(1,42)=9.9$ $p=0.003$). Hence the data for both sexes were reanalysed to establish whether prenatal treatment affected them both to the same extent.

There was a significant main effect of prenatal treatment on male pup activity (see Fig. 3.5a); the activity of treated pups was enhanced compared to controls ($F(1,22)=4.34$ $p<0.05$).

In the case of female pups, the effect of exposure to Al *in utero* on activity was in the opposite direction (see Fig. 3.5b); pups having been exposed to Al *in utero* were less active than controls ($F(1,20)=5.63$ $p<0.03$). Similarly, pups reared by treated mothers crossed fewer squares than their control counterparts. There was no significant interaction between prenatal and foster mother treatment. Thus, there was a differential effect of prenatal exposure to $Al_2(SO_4)_3$ on male and female activity scores at weaning.

Fig. 3.6 shows the median time taken to contact a novel object placed in the centre of the openfield at the end of the 5 minute activity test. The presence of a prenatal effect can be seen; **Tt** pups took less time to make contact and **Cc** pups the longest, although this was not significant. There was no correlation between scores in the activity test and latency to contact the object, illustrating that pups were not making contact by merely bumping into it.

Fig. 3.5a

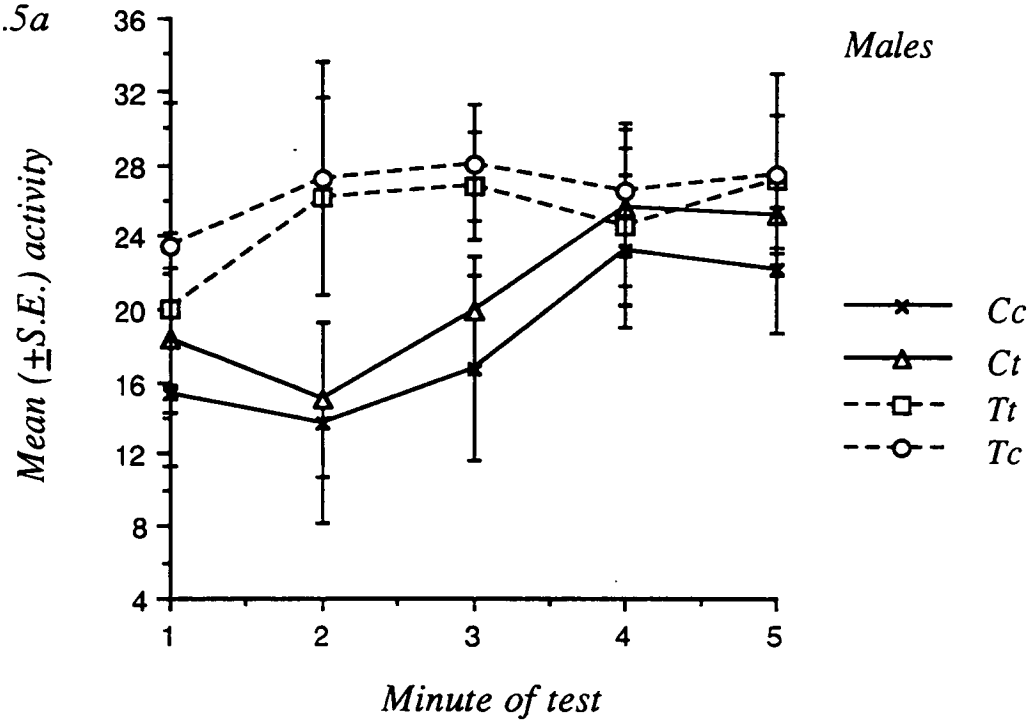


Fig. 3.5b

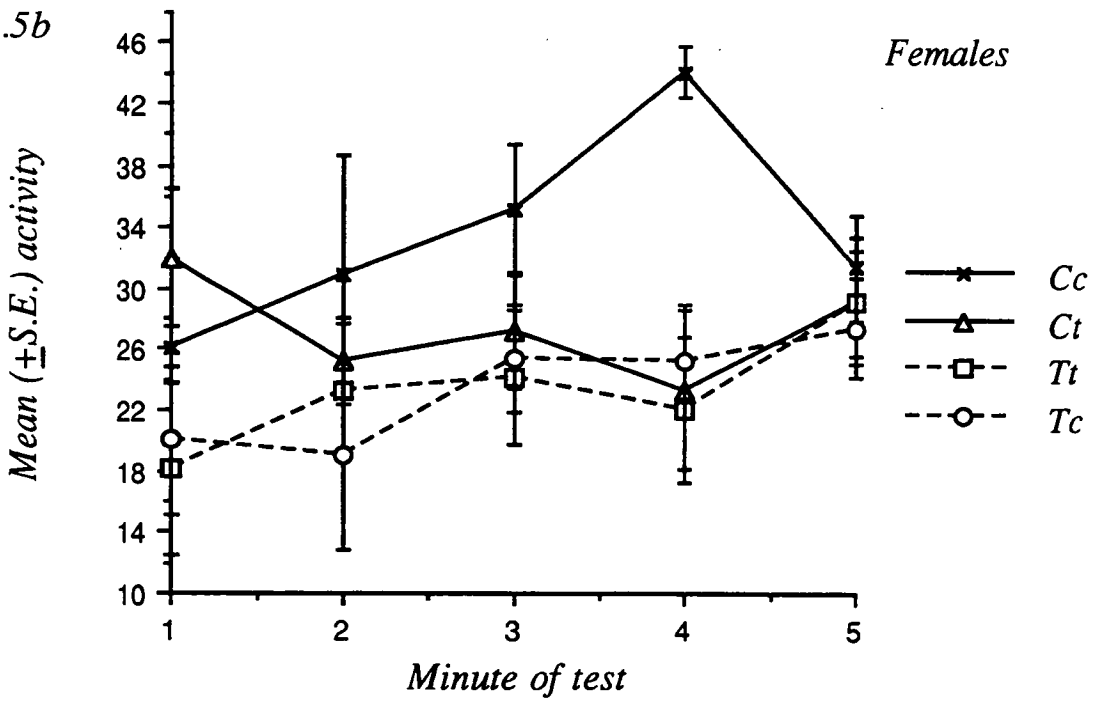


Fig. 3.5: Effect of prenatal exposure to aluminium sulphate (200mg/kg, Gd10-13) on the number of squares crossed in a five minute openfield test by CBA male (a) and female (b) pups.

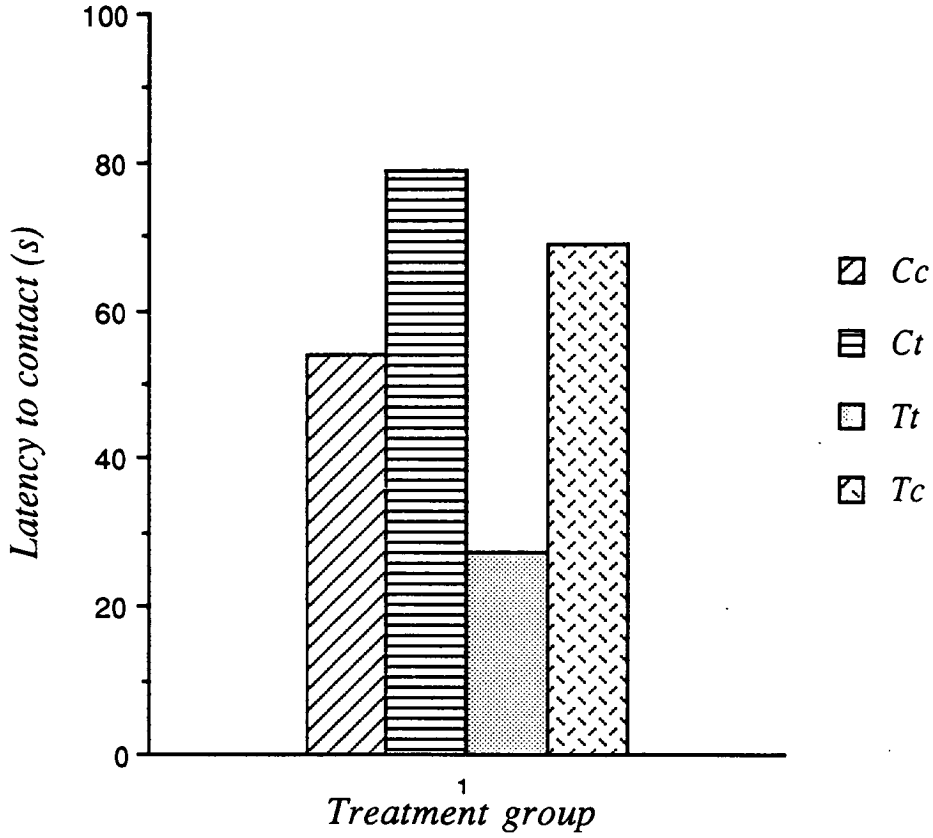


Fig. 3.6: Effect of prenatal exposure to aluminium sulphate (200mg/kg/Gd10-13) on the time taken by CBA pups to make contact with a novel object.

3.4.9 Maze test

Only data from those mice which fulfilled the criterion of entering 8 arms out of 8 on 2 consecutive days were used for the analysis of the radial arm maze tests. The ability to learn the maze is reflected by :-

1) the reduction in the number of trials needed to enter all 8 arms (Fig. 3.7a) and

2) an increase in the number of correct responses (see Fig. 3.7b).

There were no significant effects of prenatal or foster mother treatment for either of these measures. Similarly, there was no difference in the time taken to move around the maze suggesting that there was no impairment of motor capacities.

A greater proportion of control animals reached criterion (10 out of 13, 77%, 5 in the **Cc** and **Ct** groups) than treated mice (6 out of 11, 55%, 3 in the **Tt** and **Tc** groups) although chi-square analysis did not show this to be significant. Control mice took fewer days to complete the task ($\bar{x}$=6.6($\pm$0.99) days) compared to treated subjects ($\bar{x}$=7.33($\pm$1.20) days).

By retesting the animals in the maze after an interval of 5 weeks it was hoped to be able to assess the effects of treatment on the ability of subjects to recall a task in which they had been previously trained. The control group had a median of 8 visits during the last day of the first session and a median of 10.5 after the first trial of the second session. The treated animals were no worse at recall as their median number of visits was also 8 on the last day of

Fig. 3.7a

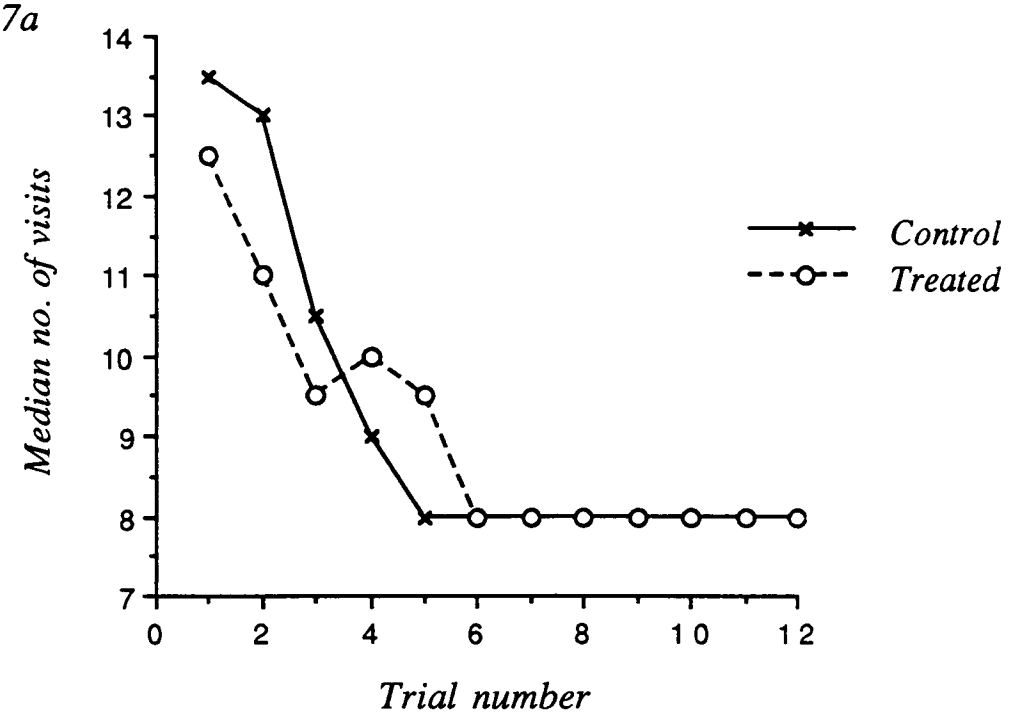


Fig. 3.7b

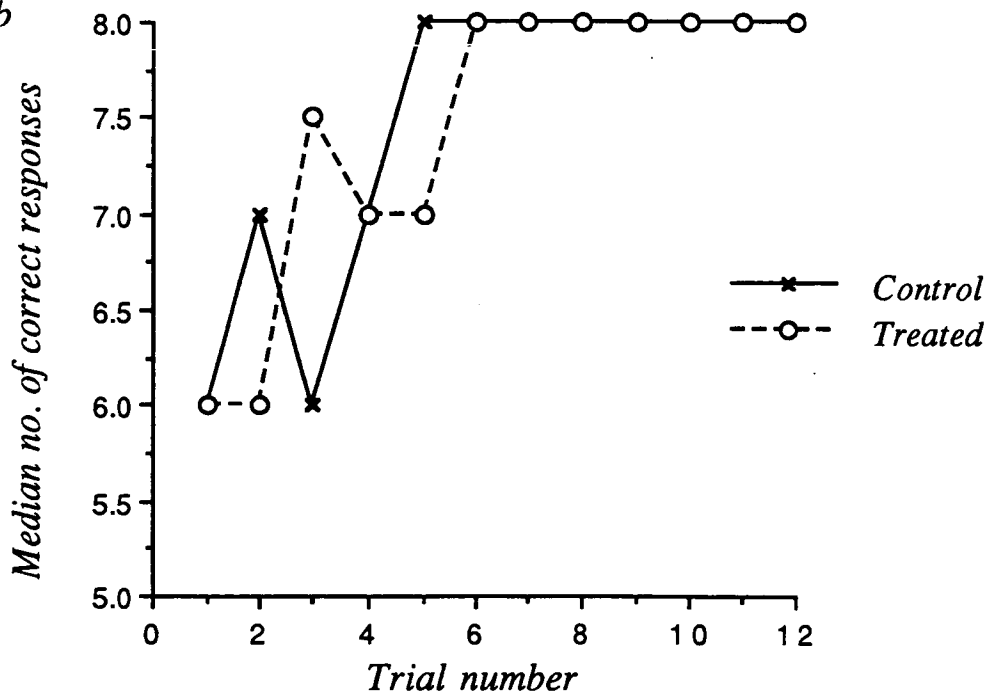


Fig. 3.7: Effect of prenatal exposure to aluminium sulphate (200mg/kg, Gd10-13) on performance of CBA males in an 8-arm radial maze.

session 1 and 9 on the first trial of session 2. There is no statistical significance between the groups in this respect.

Testing for an individual stopped once it reached criterion. Thus the score allocated to it for the subsequent days, required for other subjects to reach criterion, was always 8. This is a weakness of the method, as some subjects received more experience in the maze than others, reflecting the highly diverse intra- and intergroup variances in the level of learning ability. However, exposing all subjects to the maze until the majority of subjects have learnt it, may have introduced the problem of overtraining.

As expected, subjects required fewer trials to learn the maze during the second session and there were clear indications of savings. However, not all the subjects who completed the task in the first session had similar success in the second. 6 out of the original 10 control animals reached criterion in the second session compared to 3 of the initial 6 treated mice.

3.4.10 Adult activity

Only adult female activity scores were analysed at week 22, because a sample of male mice were sacrificed at week 17 for neurochemical analysis. The small number of male subjects remaining (**Cc** n=4, **Ct** n=4, **Tt** n=3, **Tc** n=3) was insufficient for statistical purposes. Hence, it was not possible to examine whether the differential effects of prenatal treatment on male and female activity at weaning persisted into adulthood.

There were few group differences in female adult activity levels during day 1 of testing. Group differences were more obvious

on days 2 and 3. On day 2, analysis of the number of squares crossed per minute of the test revealed a significant group difference in minute 2 ($H(3)=8.898$ $p<0.03$) and a marginally significant difference during minute 3. Again, during these minutes the maternal effect was evident, pups having been raised by Al-treated mothers having lower activity scores than pups fostered to control females. Similarly, there was a marginally significant difference in the total number of squares crossed on day 2. **Cc** females crossed a median of 174.5 squares during the 5 minute test period compared to a median total of 89 for **Tt** females.

During minute 1 of day 3 **Cc** mice crossed a median of 48 squares and **Tt** only 21 ($H(3)=10.35$ $p<0.02$). Likewise it follows that there was a group difference in the total number of squares crossed, although this result was only marginally significant.

Tt female mice took longer (median=59s) to enter the openfield at the beginning of the activity test on day 1. However this was not the case on any other day. There was also no significant difference in the time taken to contact the novel object placed in the centre of the openfield on day 4.

3.4.11 ChAT activity

Tables 3.1 and 3.2 summarise the activity levels of choline acetyltransferase (ChAT) in the different brain regions from postnatal weeks 3 to 44 for mice whose mothers were exposed to $Al_2(SO_4)_3$ during gestation and for their corresponding controls. The most consistent findings within these brain regions are that ChAT activity levels varied as a function of prenatal treatment but

also declined with age. Levels of ChAT activity were found to increase between weeks 3 and 17 in the 4 regions examined and fell in all areas between 17 and 34 weeks, although the extent to which it declined varied with brain region. For example, the ChAT level in the control hippocampus fell by 62% between weeks 17 and 34, whereas the midbrain's reduction was 18%. The changes in ChAT activity between weeks 34-44 were not as consistent as at earlier ages. In the case of the hippocampus and the cerebellum, ChAT levels were higher at week 44 than at 34 weeks of age.

Disparities in levels of ChAT may exist at 3 weeks of age because the cholinergic system does not mature completely until around the third postnatal week (Campbell, Lytle and Fibiger, 1969; Alleva and Bignami, 1985). However when one compares the ChAT levels at 44 weeks with those at 17, ChAT activity in all regions had declined with age. ChAT levels in treated brains increased between weeks 3 and 17 and similarly declined by 34 weeks of age. Again the loss in ChAT activity varied with brain region. ChAT levels declined with age between weeks 34 and 44 in four of the six regions but increased in the cerebral cortex and to a greater extent in the hippocampus. Treated ChAT levels decreased to a lesser degree with time than controls although the levels to start with (at week 17) were lower.

In the cerebral cortex, cerebellum and hippocampus the direction of change in ChAT levels between the control and treated subjects was the same at each time point, treated brains having a reduced level compared to controls. In the cerebral cortex the level of ChAT activity was 42% higher in the controls compared to treated animals at week 34. At week 44, the treated levels were still lower

Table 3.1: Effect of in utero exposure to aluminium sulphate (200mg/kg, i.p.) during days 10-13 of gestation on the level of choline acetyltransferase (ChAT) activity in different brain regions of CBA mice.

Brain region	3 weeks Control	3 weeks Treated	17 weeks Control	17 weeks Treated
Cerebral cortex	213.4 ± 9.3	210.9 ± 4.8	440.3 ± 2.6	328.9 ± 8.3
Cerebellum	43.3 ± 0.7	50.7 ± 3.1	98.5 ± 2.7	64.9 ± 1.9
Hippocampus	744.4 ± 7.6	580.2 ± 8.2 *	1015.6 ± 31.0	917.9 ± 3.6
Midbrain	N.D.	N.D.	793.3 ± 13.1	856.6 ± 3.9 *
Hypothalamus	N.D.	N.D.	665.7 ± 19.9	573.9 ± 26.7
Striatum	459.6 ± 9.7	251.5 ± 5.2 *	2141.7 ± 52.7	1871.4 ± 43.9 *

Activity is expressed in picomoles/ mg protein/ minute and each result represents the mean (±S.E.) of six determinations.
* $p<0.05$
N.D. = not determined

Table 3.2: Effect of in utero exposure to aluminium sulphate (200mg/kg, i.p.) during days 10-13 of gestation on the level of ChAT activity in different brain regions of CBA mice at 34 and 44 weeks of age.

Brain region	34 weeks Control	34 weeks Treated	44 weeks Control	44 weeks Treated
Cerebral cortex	309.4 ± 22.0	180.6 ± 1.4 *	247.0 ± 5.3	215.4 ± 2.5 *
Cerebellum	50.9 ± 1.1	46.5 ± 0.9 *	73.2 ± 1.1	45.6 ± 1.0 *
Hippocampus	381.1 ± 18.1	306.7 ± 1.4 *	567.2 ± 6.0	545.5 ± 10.0
Midbrain	649.4 ± 7.8	552.1 ± 6.3 *	505.7 ± 15.0	439.8 ± 3.8 *
Hypothalamus	530.9 ± 2.1	433.8 ± 6.3 *	247.5 ± 2.4	342.2 ± 9.0 *
Striatum	1047.7± 24.3	995.0 ± 7.0	431.7 ± 8.4	728.2 ± 16.0 *

Activity is expressed in picomoles/ mg protein/ minute and each result represents the mean (±S.E.) of six determinations.
* $p<0.05$
N.D. = not determined

but the divergence was not as marked (13%). The reduction in the disparity between the two groups is probably due to the fact that ChAT levels were falling with age in the control group in any case, so that the effects of the initial Al insult were confounded by age-related changes. The hippocampus expressed the highest levels of ChAT. At 17 weeks the difference between the control and treated groups was only 10% in this region. Levels of ChAT were lowest in the cerebellum. They peaked at 17 weeks and thereafter declined. Significant differences occurred between the control and treated groups during weeks 17, 34 and 44, the greatest difference occurring at 44 weeks (38%).

The mid-brain and striatum were the exceptions. In the mid-brain ChAT activity was raised in the treated brains at 17 weeks, a difference of 8%, but by the older ages levels in the treated group were reduced compared to controls. The overall loss in ChAT activity was greater in the treated group, being almost halved at 27 weeks. Very high levels of ChAT were found in the striatum which may have resulted from this region containing the nucleus magnocellularis which has the richest source of ChAT and which was not dissected separately in this experiment. ChAT activity was reduced in treated brains at 17 and 34 weeks but significantly increased (41%) at 44 weeks.

3.5 SUMMARY OF RESULTS

Exposure of pregnant CBA females to injected Al over a limited period of gestation (Gd10-13), caused a reduction in maternal weight gain but did not affect breeding performance.

Pups exposed to Al *in utero* had a reduced body weight at birth compared to controls; this difference persisted only for those pups fostered to treated mothers. This effect on body weight persisted into adult life in the case of female animals. Al-treated pups showed delays in the attainment of an adult-like response in several of the Fox tests. Although Al did not affect the overall expression of pup behaviours, it altered the frequency and duration of several behaviours on certain days. Activity scores at weaning were both treatment- and strain-dependent. Females exposed to Al were hypoactive compared to controls whilst the effect on males was to increase activity levels. This effect on female activity was still present at 22 weeks of age. Al had only slight effects on cognitive ability as assessed in the radial maze. Treated animals showed deficits of ChAT activity in several brain regions.

Effect of exposure of CBA mice to injected aluminium sulphate over five days of treatment.

3.6 EXPERIMENT 2

3.6.1 Background

The effects of gestational exposure to injected aluminium, as described in experiment 1, were patchy but clear enough where they took hold. The treatment period was restricted in experiment 1 due to the hardening of the skin around the injection site. In this second experiment a new batch of mice were given an extended treatment period of five days.

3.7 GENERAL PROCEDURES

3.7.1 Treatment

Plugged CBA females were injected i.p. with 200mg/kg body weight $Al_2(SO_4)_3$ but the dosing period was extended to five days from Gd10 to Gd14 inclusive.

Most of the procedures used in this experiment have been described previously (see section **2.2**).

3.7.2 Maze testing

The same food deprivation procedure was undertaken as described in *2.2.3a*. However, in this experiment the subjects were

tested twice a day for 6 days (in the morning and afternoon) instead of only once a day. A test for recall of maze learning was not measured in this experiment.

The neurochemical data presented in tables 3.1 and 3.2 suggests that exposing subjects to Al causes alterations in the cholinergic system, a result which has also been reported in other studies (McGurk, Levin and Butcher, 1988). Because of the well-established role of acetylcholine in learning ability, such deficits might be expected to result in equivalent defects in the maze learning tests. However, experiment 1 (**3.2**) did not reveal any such effects, perhaps because the task was not sufficiently challenging to expose any differences between the groups. For this reason, an extra treatment is introduced here in the maze testing. All subjects were challenged with a muscarinic cholinergic antagonist, scopolamine (Brown, 1990), with the aim of augmenting any group differences.

When the majority of subjects had reached the criterion of entering 8 arms in 8 visits, they were divided into two groups. Each animal was removed from the home cage, injected i.p. with either scopolamine hydrochloride (0.5mg/kg body weight), or saline. The subject was then placed in a new cage for 15 minutes, the time expected for scopolamine to take its effect, prior to being tested in the radial maze, following the same procedure as before (see *2.2.3b*). On completion of the session, the subject was returned to its home cage and given access to food. The challenge with scopolamine took place on three alternate days (Mon, Wed, Fri), with maze testing occurring once on each of the intervening days (Tues, Thurs).

3.8 RESULTS

From the outset, there was a low number of plugged females and only five mothers in the control and four in the treated group continued to term. It was not possible to ascertain with confidence whether this small number of litters resulted solely from the effects of treatment. However, as both control and treated females were similarly affected this suggests that the lowered number of litters was caused by the reduction in the number of females who were actually plugged, rather than a direct effect of exposure to Al. These mice were mated in February which has proved to be a time of year when matings are harder to obtain in Edinburgh.

3.8.1 Estimated level of aluminium exposure

Over the five days of treatment, pregnant CBA mice received a total of 2.01mg of elemental Al.

3.8.2 Breeding performance

Treating pregnant CBA mice with 200mg/kg $Al_2(SO_4)_3$ during gestation days 10 to 14 did not affect the length of gestation (control n=5 $\bar{x}$=21.0($\pm$0.32), treated n=4 $\bar{x}$=20.25($\pm$0.25) days) or litter size (control $\bar{x}$=5.6($\pm$0.6) treated $\bar{x}$=4.75($\pm$0.85)). As in experiment 1, exposure to $Al_2(SO_4)_3$ had a diminishing effect on maternal weight gain between gestation days 10 to 14, although this did not reach significance. The total mean weight gain of control

mothers during the treatment period was greater than that of treated mothers (control $\bar{x}$=2.96(±0.27)g, treated $\bar{x}$=0.7(±0.4)g $t(5)$= 4.69 $p<0.006$).

3.8.3 Pup weight

As before, exposure to $Al_2(SO_4)_3$ *in utero* significantly reduced the birth weights of treated pups (control n=27 $\bar{x}$=1.33(±0.03)g, treated n=19 $\bar{x}$=1.05(±0.03)g, $t(39)$= 6.87 $p<0.0001$).

Achieving a total split-litter cross-foster design was made possible only by including more control pups. This resulted in one litter being made up of four fostered control pups only, hence it could not be included in the testing. Within those litters that were cross-fostered, 2 treated mothers had killed 1 fostered treated pup by Pd3. This was the only group within which postnatal mortality occurred. There remained a total of 26 pups; **Cc** n=7, **Ct** n=9 and n=5 for both the **Tt** and **Tc** groups.

During the preweaning period differences in the rate of growth were analysed as opposed to the actual weights because the litters were not all born within the same 12 hour period. The weight gains of the **Tt** group were not normally distributed so weight gain was analysed using the Kruskal-Wallis one-way ANOVA with multiple comparisons. Fig. 3.8 shows the median weight gain for male and female pups from days 6 to 21 postpartum. There was a significant group difference in weight gain between postnatal days 3 to 6 ($H(3)$=14.89 $p<0.002$) and between days 6 and 9 ($H(3)$=12.36 $p<0.007$). **Cc** pups gained significantly ($p<0.05$) more weight than

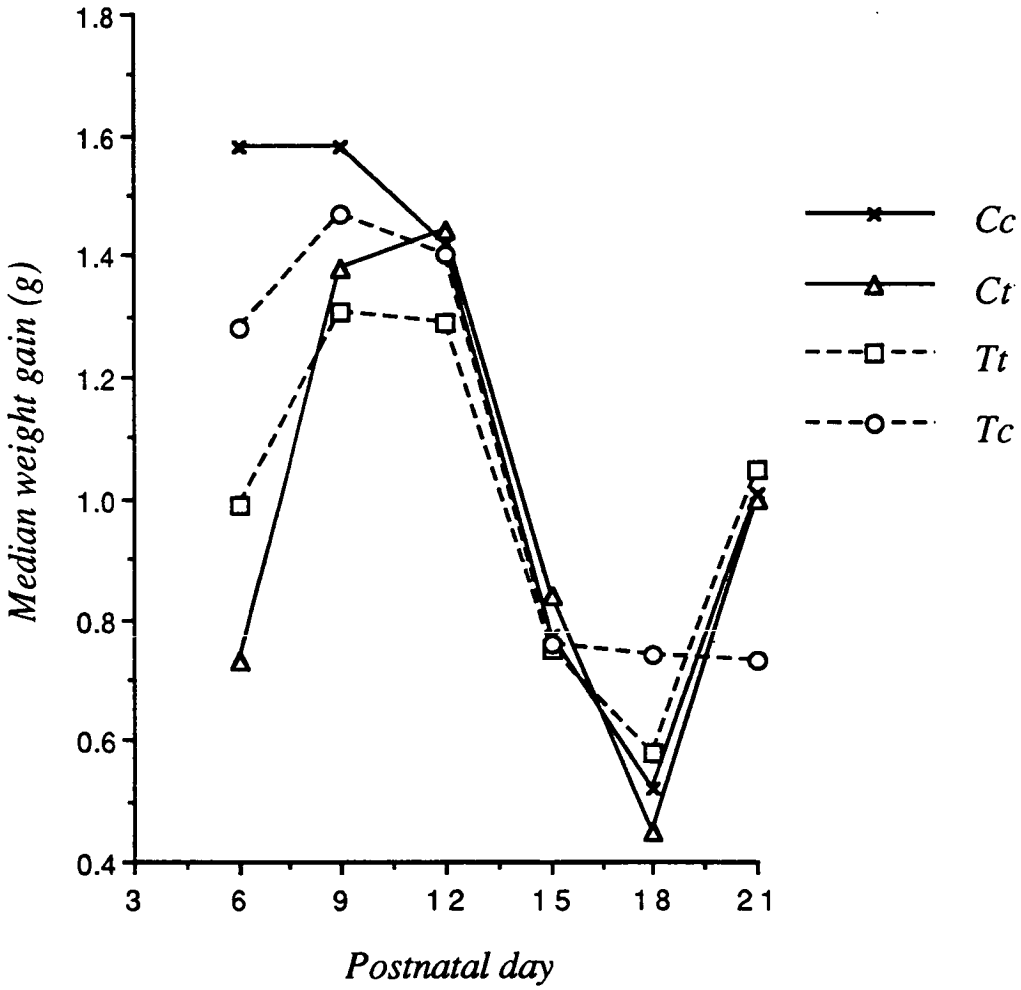


Fig. 3.8 : Effect of prenatal exposure to aluminium sulphate (200mg/kg, Gd10-14) on CBA pup body weight gain.

the other groups during these days. From Pd9 onwards all pups gained weight at a comparable rate.

3.8.4 Pup behaviours

As expected the frequency and duration of bouts of each behaviour increased with age. The following pup behaviours showed differences between the experimental groups:-

Crawling - Prenatal treatment significantly affected the frequency ($F(1,16)=16.47$ $p<0.001$) and duration ($F(1,16)=13.04$ $p<0.003$) of bouts of *crawling*, control pups exhibited a greater number of bouts and of longer duration. There was also an interactive effect of day and prenatal treatment on the frequency of *crawling* ($F(4,64)=3.03$ $p<0.04$). The trend towards increased activity in the case of control pups was also found in the analysis of square crossing, although this did not reach significance.

Headup - There was a significant interactive effect of day, prenatal treatment and foster mother treatment on the frequency of *headup* behaviour, pups belonging to the **Ct** and **Tc** groups exhibited an increase in *headup* behaviour on certain days ($F(5,85)=2.86$ $p<0.05$). The duration of these bouts was significantly affected by prenatal treatment ($F(1,17)=8.73$ $p<0.009$), treated pups spent more of their time involved in this behaviour.

Rearing - Fig. 3.9a illustrates the obvious treatment effect on the frequency of bouts of *rearing*. Pups exposed to Al prenatally had a

Fig. 3.9a

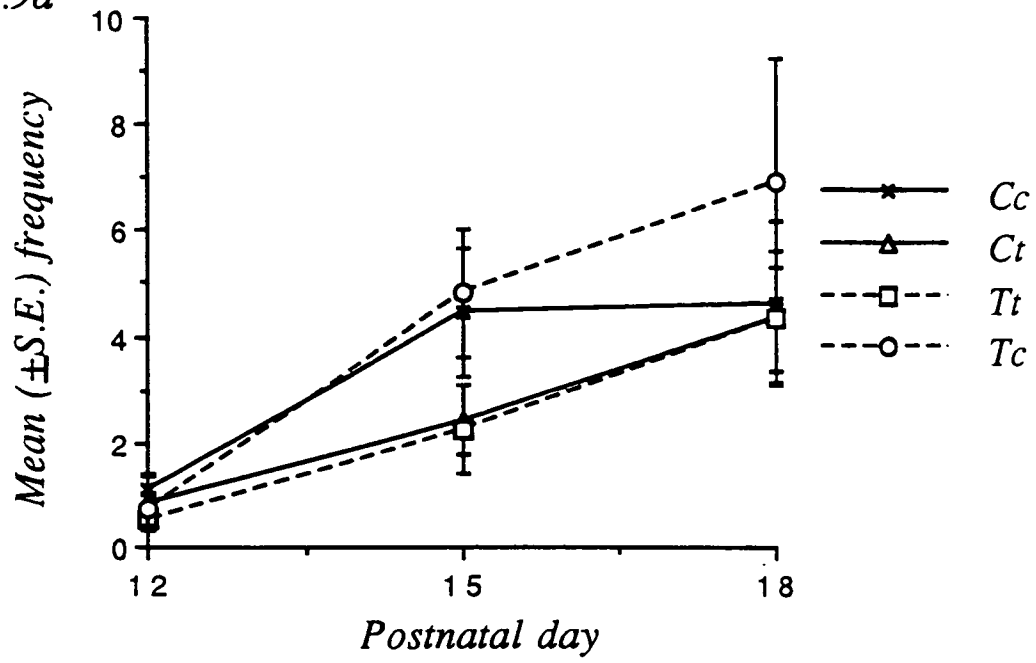


Fig. 3.9b

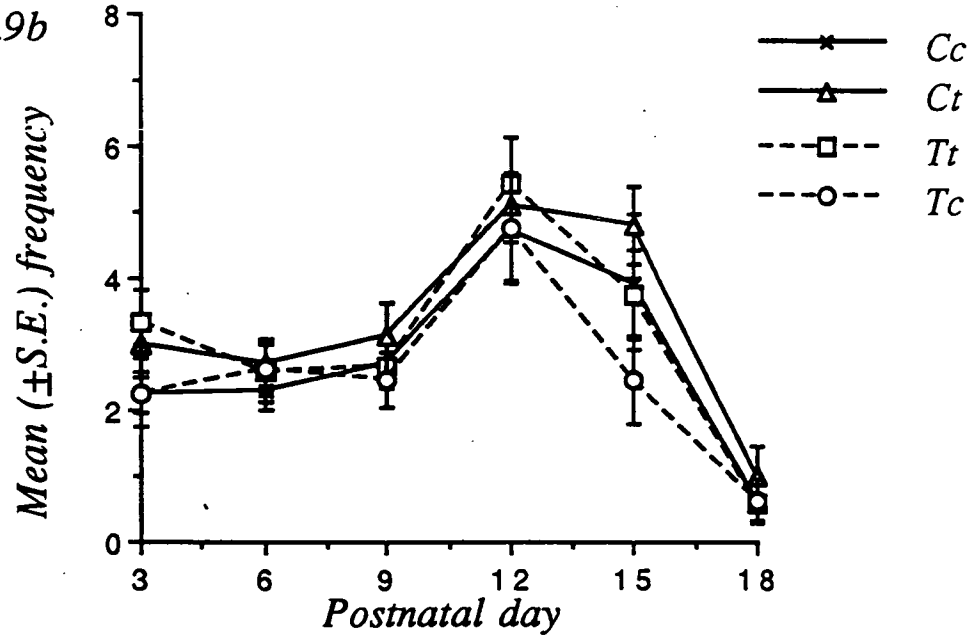


Fig. 3.9: Effect of prenatal exposure to aluminium sulphate (200mg/kg, Gd10-14) on CBA pup (a) rearing and (b) still behaviour.

diminished number of bouts ($F(1,18)=6.77$ $p<0.02$). There was a significant interactive effect of day and foster mother treatment on frequency ($F(2,36)=4.74$ $p<0.02$), pups fostered to control mothers exhibited more bouts of *rearing*.

Edgeon - There were no effects on the frequency of *edgeon* behaviour but Kruskal-Wallis one-way ANOVA of the duration of time spent rearing against the wall, revealed a significant group difference on Pd15 ($H(3)=9.19$ $p<0.03$). There was a significant difference between **Cc** and **Tc** pups ($p<0.05$).

Still - There was a significant effect of prenatal treatment on the frequency of bouts of being *still* ($F(1,17)=7.96$ $p<0.02$), treated pups showed a greater number of bouts as shown in Fig. 3.9b. Day and foster mother treatment significantly interacted to affect the frequency of bouts of this behaviour ($F(4,68)=3.14$ $p<0.04$), taken overall pups fostered to treated mothers displayed an increased frequency of being *still*. There were no effects on the duration of bouts of *still* behaviour.

3.8.5 Fox tests

Similar developmental delays in the Fox tests were found following five days of treatment with Al as occurred after the four days exposure of the previous experiment. Treated pups were slower to attain a mature response in *swift righting* on Pd12 ($X^2(3)=9.857$ $0.02>p>0.01$); in *cliff aversion* on Pd15 ($X^2(3)=9.1$ $0.05>p>0.02$); and in *pole grasping* on Pd18 ($X^2(3)=14.942$ $0.01>p<0.001$); fewer

pups from the **Tt** group acquired an adult response in these particular tests. Performance on these Fox tests were not correlated with weight gain.

3.8.6 Activity test at weaning

There were no significant differences in activity at weaning between the sexes in this sample of pups. Likewise there were no significant group differences in the number of squares crossed during any minute of the test when male and female pups were analysed together or in the overall total number crossed. The number of crossings varied significantly with minute of test ($F(4,72)=3.80$ $p<0.02$), pups were more active during minute 1 but thereafter they showed within-session habituation.

Ct pups took the longest time to contact the novel object placed in the centre of the openfield at the end of the activity test (median=63s) and **Tc** the least time (median=32s), although this difference was not significant.

3.8.7 Maze test

In the maze testing for this experiment, subjects were trained twice a day for six days rather than once a day. It was hoped that this would reduce the length of time over which the animals had to be food deprived but still maintain motivation to learn the task. Animals were not removed from the testing schedule until the majority of mice had completed the task, thus equalising each

subject's experience of the apparatus. As the sample size was small, subjects were not divided with respect to foster mother treatment.

By trial 12 only 67% (6 out of 9) of the control animals and 83% (5 out of 6) of the treated mice had reached criterion. Control subjects did not require as many trials to complete the task as did treated subjects (control median=4.5, treated median=9.0). There was a slight difference in the time taken per arm entry by control animals between the morning and afternoon training sessions during day 2. This may have been related to differences in hunger between the two training trials. The length of time without food, between taking the food away after the afternoon session and the morning trial, was greater than the time between the morning and afternoon trials. No differences as a result of time of day of testing occurred on any other measure taken.

Increasing the dose period from four to five days did indeed seem to cause a greater disruption in maze performance. Fig. 3.10a and b show the number of arms visited during each trial and the number of correct responses in the first 8 entries. Treated animals required a greater number of arm entries to complete the task during trials 1 to 5, although these differences did not reach significance. Treated animals also made significantly fewer correct responses during trial 3 (control median=7.5, treated median=6.0, U=27.0 p=0.02) and to a lesser extent during trial 4 (control median=8.0, treated median=6.0, U=25.0 $p<0.05$). The time taken to complete the test did not differ significantly between groups, thus there were no differences in the subjects' overall ability to move around the maze.

Statistical analysis of the scopolamine challenge data was not undertaken due to the small sample sizes. However, the results of

Fig. 3.10a

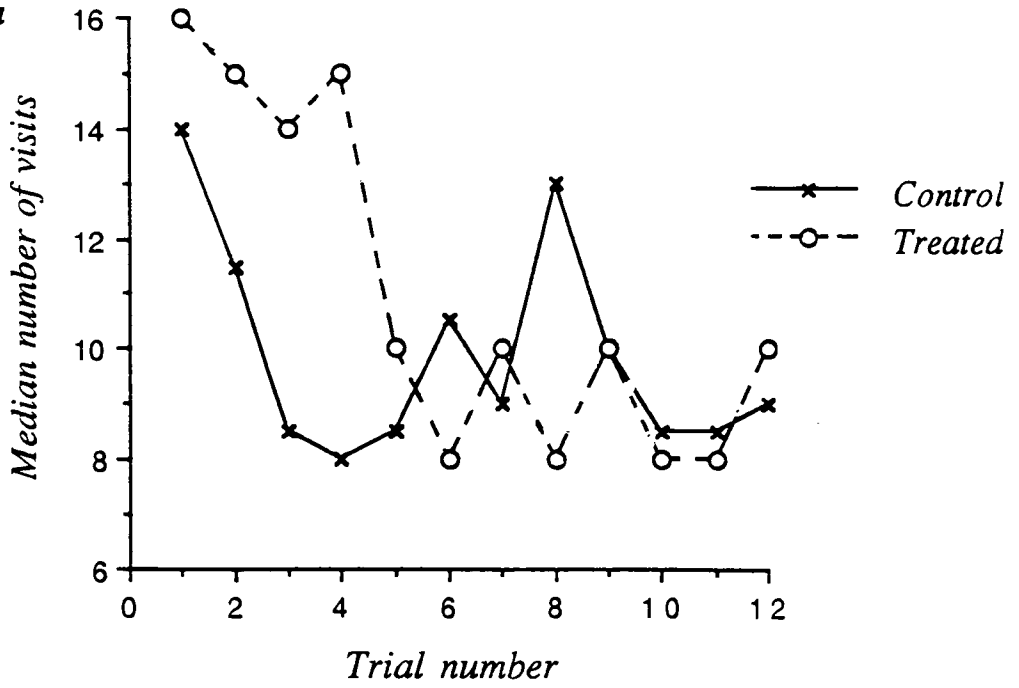


Fig. 3.10b

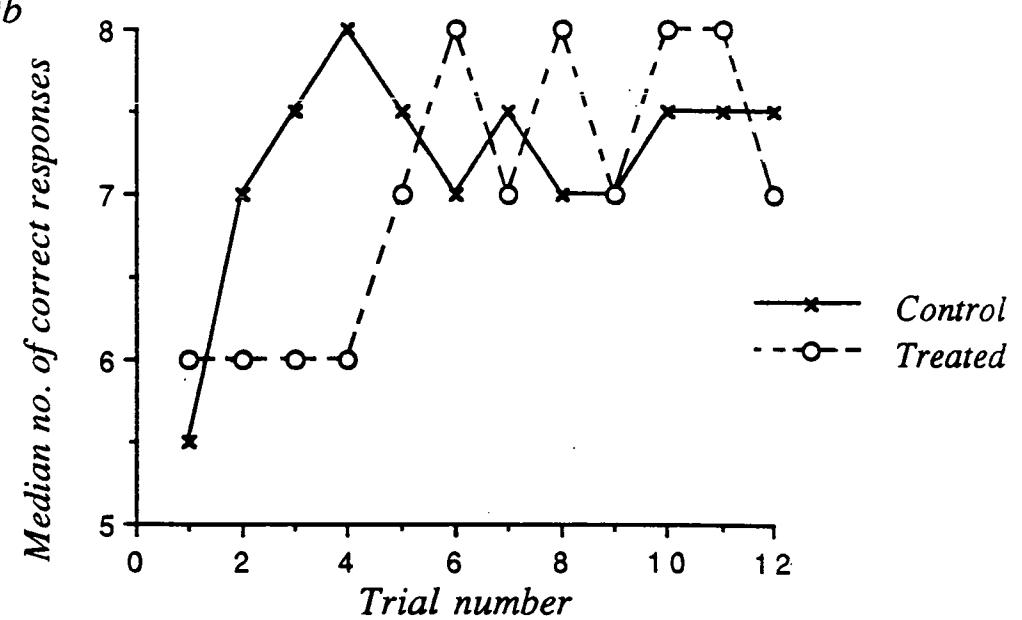


Fig. 3.10: Effect of prenatal exposure to aluminium sulphate (200mg/kg, Gd10-14) on performance of CBA males in an 8-arm radial maze.

this preliminary experiment suggest that scopolamine had the predicted effect on control animals i.e. caused a disruption of performance in the maze but this took longer to manifest in the treated animals. The intermediate test days, when subjects were tested in the maze but not exposed to scopolamine, indicates its short-acting nature as most subjects recovered to their baseline level of performance.

There were insufficient mice to undergo adult activity tests.

3.8.8 ChAT activity

A sample of control and treated male subjects was sacrificed at 14 weeks of age following the maze test for neurochemical analysis. The levels of ChAT activity in each brain region is given in table 3.3.

Analysis of ChAT activity was undertaken only at the one age of 14 weeks. The direction of change in activity was dependent on brain region. The level of ChAT in the cerebral cortex, hippocampus and the nucleus magnocellularis of control brains was greater than that measured in treated brains. The difference was most pronounced in the nucleus magnocellularis (40%). This direction of change was similar to that found in 17 week old mice in experiment 1 (see Table 3.1). Conversely, treated hypothalamus and striatum contained higher levels of ChAT activity than controls, differences of 35 and 25% respectively.

Table 3.3: ChAT activity in CBA males whose mothers were exposed to Al (200mg/kg body weight) by i.p. injection during gestation days 10-14 .

Brain Region	Treatment	Mean(±S.E)	*t*-value	p-value
Cerebral Cortex	Control Treated	440.3± 2.6 328.9±18.3	6.02	<0.002
Cerebellum	Control Treated	61.2± 4.6 57.3± 2.2		N.S
Hippocampus	Control Treated	618.5±50.5 471.7±15.3	2.78	<0.04
Midbrain	Control Treated	573.8±30.5 618.7±12.1		N.S
Hypothalamus	Control Treated	218.6± 7.4 335.4± 5.8	-12.45	<0.0001
Nucleus Magnocellularis	Control Treated	2066.2±28.3 1198.5±95.9	8.68	0.0003
Striatum	Control Treated	456.2±11.5 605.6± 8.7	-10.35	<0.0001

Effect of gestational exposure of C57BL/6J mice to injected aluminium sulphate.

3.9 Experiment 3

As discussed in chapter 1, the susceptibility of an individual to a particular agent is greatly influenced by its genotype (see **1.2***v*). To test this with regard to possible behavioural teratogens, plugged females from the second inbred strain of mouse, C57BL/6J, were injected i.p. with either 200mg/kg body weight $Al_2(SO_4)_3$ or the equivalent volume of saline on Gd10 to Gd13 inclusive.

Fifteen control and 16 treated C57 females were found to be pregnant. However, these mothers, who were considered to be pregnant as judged by the overall weight increase during gestation, nevertheless failed to produce any viable young. Only blood and pup carcasses could be seen in the cages on the expected day of birth. This was also the case for controls. Reducing the exposure period to one day only (Gd10) resulted in five mothers in each treatment group producing viable young, although cross-fostering was not possible due to the small number of pups born and asynchrony in their times of birth.

It is not clear whether the failure to produce sufficient viable young resulted form a direct effect of exposure to Al, or from the fact that, from the outset, an insufficient number of females had actually been plugged.

3.10 DISCUSSION

In utero exposure of CBA females to $Al_2(SO_4)_3$ had a direct effect on the developing foetus resulting in lower birth weights of offspring from treated mothers, after four or five days exposure. This direct effect on body weight was transient as no significant effect of prenatal treatment was found after Pd3. What is clearly apparent from experiment 1 (**3.2**) is that from Pd6 onwards the previous treatment received by the foster mother during her pregnancy, continued to influence a pup's physical development. Fostering a control pup, which had had no contact with $Al_2(SO_4)_3$ *in utero,* to a treated mother reduced growth compared to control pups reared by control mothers. On the other hand, fostering a treated pup, which starts from a lower birth weight, to a control mother enables the pup to overcome this initial disadvantage and to restore its physical development to normal.

The reduction in birth weight of rat pups exposed *in utero* has been reported previously. Benett, Persaud and Moore (1975) found an increase in the number of deaths and a diminution of body weight in both mothers and their offspring following i.p. injections of $AlCl_3$ at the same dose as used in this experiment (200mg/kg). Similarly, the offspring of BALB/c mice had a reduced birth weight after i.p. exposure to $AlCl_3$ during gestation days 7 to 16 (Cranmer, Wilkins, Cannon and Smith, 1986).

The maternal effect on weight persisted into adult life for females, suggesting that the effects of exposure to Al are greater on female offspring and are not transient. The lack of significant effect on adult male weight may have resulted from the variance within the

small sample but Al-exposed male mice may be better able to recover from the initial prenatal insult.

This maternal effect on pup growth is not obvious in experiment 2 (**3.6**) when CBA pregnant mice were exposed to Al for five days but it is difficult to interpret these results satisfactorily because of the small sample of treated pups. Loss of litters occurred in both control and treated groups. Thus one can not conclude unequivocally that Al alone caused the reduction in birth weight. However, there was a decrease in weight gain in the surviving treated offspring until Pd9. After this, exposed pups gained weight at a rate comparable to that of controls although they remained lighter throughout. This suggests that the treated pups could not completely overcome their reduced birth weight.

The nature of this maternal effect is unclear and at present one can only speculate on possible mediating factors. The postnatal maternal influence may result from retention of Al within the mother's body which is subsequently released to the pups via milk. This could occur if Al became bound to maternal tissues during gestation. Alternatively, it is possible that the injected Al binds to tissue surrounding the injection site and is slowly released during actual treatment and for a few days following. Repeated administration of an agent extends the period over which the subject is exposed. Hence, Al could become available to the newly fostered pups via the dam's milk. Control pups, which had had no prenatal contact with Al, may have been exposed to it during the early postnatal days. Dobbing (1968) has shown that the rat brain is most vulnerable to experimental insult during the suckling period. Indeed, Yokel (1985) found that it was not only offspring of mothers

exposed to 400 μmol/Al/kg which gained less weight, but also control offspring fostered to these treated does. In the experiments reported in this chapter, Ct pups could have been exposed to Al only during suckling. Moreover, pups exposed to Al *in utero* will be further exposed during this time. Even if postnatal exposure to Al only lasts for 1-2 days, this may be sufficient to affect the developing pup. Yokel and McNamara (1985) injected lactating rabbits with AlLact and found that seven days after the last injection 12% of the total injected Al was still present in the area of the injection site. It would be interesting to isolate tissue from the injection site at different ages following completion of treatment, and analyse it for Al content.

The solubility of Al is greatly increased when it is complexed to organic compounds e.g. citrate (Slanina, Frech, Ekström, Lööf, Slorach, and Cedergren, 1986). Within the mother's body Al may bind to lactate in the milk. If Al does pass to the infant in the milk, this may be important if the gut epithelium of the perinatal infant is more susceptible to agents present in the mother's milk than is the postnatal pup. In addition, Al present in the maternal tissues may influence the quantity or quality of milk available to the offspring. This possibility remains hypothetical as neither milk output nor its Al content were measured. However, Yokel and McNamara (1985) found only low levels of Al present in the milk from does which had been exposed to AlLact during lactation.

Other studies have definitely shown that Al administered to the mother can pass to the foetus. Cranmer, *et al.*, (1986) found the Al content within the placenta of mice injected with $AlCl_3$ at a dose of 100mg/kg/day from Gd7-16, to be ten times higher than control

values and the total body Al in the foetuses of these mothers was three times higher. Thus, injected Al is available to pass via the placenta to the foetus and accumulate therein. Furthermore, research has shown that Al can cross the foetal blood-brain barrier (Petit, 1988). $AlCl_3$ ingested by the rabbit resulted in an increased Al content of both the mother's milk and within the brains of suckling rabbits. Al is known to bind to albumin and transferrin within the body (Martin, 1986) and in these forms its solubility, and therefore its ability to cross membranes, is increased.

A second possible explanation for the maternal treatment effect is a direct effect of gestational Al on maternal behaviour. External influences are known to alter maternal behaviour which in turn has consequences for offspring development. Barnett and Burn (1967) found that handled pups received more maternal care on return to the home cage. Pups in the experiments reported herein were repeatedly removed and then returned. Control mothers may have given more care to their fostered young after they had been tested than did treated mothers. The contribution of alterations in maternal care to the maternal effect could be ascertained by observing maternal behaviour directly in an observational study (see chapter 5).

The young of a variety of diverse rodent species are known to emit calls within the ultrasonic range in circumstances of distress e.g. as a result of cold, isolation and hunger. Exposure to $Al_2(SO_4)_3$ during gestation may have affected the ability of treated mothers to respond adequately to the ultrasonic calls of their fostered pups. Alternatively, the treated pups themselves may have been less efficient at producing these calls. A pup previously disadvantaged by

being exposed to Al during gestation and then fostered to a treated mother, would be expected to be the most severely affected by any neglect in postnatal maternal behaviour, and this was indeed the case.

It is unclear whether the lack of viable C57 young after i.p. injections of $Al_2(SO_4)_3$, at the same dose as that administered to pregnant CBA mice, can be attributed to an increase in resorptions or spontaneous abortions during pregnancy or to an increased tendency of these mothers towards infanticide. Maternal behaviour is thought to be triggered by hormonal changes at the time of parturition (Rosenblatt and Siegel, 1983). Perhaps these changes are not sufficient in the nulliparous C57 female to elicit maternal care. Infanticide has been associated with the culling of weak or malformed young (Labov, Huck, Elwood and Brooks, 1985). Prenatal exposure to Al may alter the development of the foetus rendering it physically inferior, thus initiating natural infanticidal tendencies in the mother. Alternatively, the stress associated with i.p. administration may have affected these mothers more than the CBA strain. As pup killing occurred in both treatment groups this is a more plausible explanation.

Other aspects of development were altered by exposure to $Al_2(SO_4)_3$. Treated pups were slightly behind controls in attainment of the *righting, forelimb* and *pole grasping* reflexes. These tests are measures of the ability to orientate and of motor coordination and strength. Other agents have been found to have similar effects on these tests e.g. methylmercury and tetraethyltin (Pryor, Uyeno, Tilson and Mitchell, 1983). However, Tsujii and Hoshishima (1979) found that female CFW mice exposed to Al were accelerated in

achieving a screen climbing test compared to controls. Similarly, Donald, Golub, Gershwin and Keen (1989) reported greater fore and hindlimb strengths after weaning in pups whose mother was fed Al in the diet. It could be argued that the differences in the attainment of adult responses in this experiment may have resulted from the lower weight of the pups reared by treated mothers than from direct effects of Al on mastering this reflex. However, body weight and performance in the Fox tests did not reveal significant correlations suggesting this explanation to be unlikely.

The developmental profile of pup behaviours was similar across all groups, with the majority of pups commencing each behaviour on the same day. However group differences did emerge in some of the behaviours recorded, reflecting differences in the ontogeny of the behavioural repertoire. This was more evident after exposure to $Al_2(SO_4)_3$ over 5 days. In particular, treated pups displayed a reduction in the frequency and duration of bouts of *rearing* within the openfield and assisted *rearing* against the walls. This may have resulted from a delay in the maturation of hindlimb strength in the treated pups which is substantiated by the delay in attainment of an adult response in *forelimb* and *pole grasping*, both of which require muscular strength.

Group differences were found in locomotor activity as measured in the openfield. At weaning, female pups were more active than males. This sex difference in activity levels has also been reported in rats. Bronstein, Wolkoff and Levine (1975) found that 30-40 day old female rats were more active than males during repeated trials in the openfield. Additionally, Al-exposed females were less active than control ones. This hypoactivity was still present

at 22 weeks of age when the total squares crossed by treated female mice were significantly lower than for controls. Differences in activity were not obvious during day 1, the openfield representing a new environment to the mice, increasing their curiosity and decreasing their activity, but they were present during days 2 and 3. Conversely, treated males were more active than control ones at weaning. This further suggests that Al affects female animals to a greater extent than males.

Results from the radial maze tests certainly yield signs that Al exposure has effects on learning in this situation, although none of the differences reach a satisfactory level of statistical significance. One interpretation of the maze data is that differences resulted from impairments in motor rather than cognitive abilities. However, the time taken to complete each trial did not show any significant differences between control and treated males. Although males exposed to Al prenatally over four days required more days to complete the task, few differences in the number of arm entries were seen. Similarly, Alfano and Petit (1981) found that exposure to heavy metals led to deficits in some measures of learning ability but not in others. They exposed Long-Evans hooded female rats to lead carbonate at a level of 4% or 0.4% in their diet during postnatal days 1 to 25. The offspring were tested at 65 days of age in a 8-arm radial maze. Animals in both lead groups required more days to reach criterion than controls but did not show any differences in the number of correct choices made in the maze (Alfano and Petit, 1981). However, in the experiments described in this chapter, Al-treated males did require more arm entries to complete the maze task compared to controls, when the exposure period was extended to five

days. Unfortunately the sample size was inadequate for definitive conclusions to be drawn.

Evidence from a number of studies has shown that an intact hippocampus is essential for learning and memory (Olton and Samuelson, 1976; Olton, Walker and Gage, 1978; Olton and Papas, 1979; Becker, Walker and Olton, 1980). In section *3.4.11* (p58) control and treated animals were sacrificed after completion of the maze tests. There was only a 10% difference in ChAT levels within the hippocampus at 17 weeks of age which was not significant and this lack of any effect on ChAT may explain why only slight differences were found between control and treated subjects in maze performance. However, in section *3.8.8* (p73) it is reported that, following five days of exposure to Al, the difference in hippocampal ChAT levels between control and treated males was 24%. In this case, as noted above, slight deficits in performance by treated animals were also evident.

Scopolamine is a centrally-acting muscarinic cholinergic receptor antagonist, and thus challenging subjects with scopolamine will help to give an estimate of the contribution of muscarinic cholinergic receptors to learning. In experiment 2, there was a suggestion that differences existed between the saline- and scopolamine-treated groups but these were not substantial. It took longer for the effects of scopolamine to be manifested in the treated mice. The challenge was hindered by the small sample size and more work will be necessary before satisfactory conclusions can be drawn. Levin, Castonguay and Ellison (1987) have shown that blockage of central nicotinic-cholinergic receptors also impairs maze performance. Perhaps challenging with scopolamine antagonises the

remaining muscarinic receptors which are still present after exposure to Al, but as scopolamine does not act on nicotinic receptors, these receptors remain unaffected and are sufficient to maintain performance in the previously learnt test. Any differences between the control and treated animals in the maze may be brought out by the co-administration of scopolamine and a nicotinic receptor antagonist e.g. mecamylamine. Alternatively, altering the exact time at which scopolamine is injected may reveal greater group differences. Buresova and Bures (1982) introduced a delay between trials 6 and 7 of a 12-arm radial maze task, then injected subjects with scopolamine and found an increase in the number of errors made during choices 7 to 12. They concluded that continuous performance in the maze is not affected by scopolamine but storage of information after a delay is.

Tilson (1987) injected rats with low levels of colchicine, a neurotoxin, and found a disruption in maze performance. Animals injected with a higher dose did not show any further deficits. This finding was explained in terms of reaching a ceiling effect i.e. the maximum number of cholinergic receptors had been lost so increasing the dose had no effect. In experiment 2, exposure to Al may have caused maximum deficits in the cholinergic system so that challenging with scopolamine had no further disrupting effect on maze performance.

The picture is complicated further by the fact that, through studies involving blockage of different receptor types, other neurochemical systems have been identified as playing a role in cognitive processes. McGurk, Levin and Butcher (1988) found that exposure to scopolamine resulted in the expected deficit in the

ability to make correct responses in a maze but that this deficit was attenuated by administering the dopamine receptor antagonist haloperidol. Furthermore, Decker and Gallagher (1987) found that injection of 6-hydroxydopamine, which causes a depletion of noradrenaline, together with scopolamine led to an increased deficit in the ability to complete accurately an 8-arm radial maze. Thus, the mammalian brain which has suffered an insult may be able to initiate compensatory mechanisms to overcome any cholinergic deficit and can mediate adequate maze performance. For example, dopamine fibres from the ventral tegmental area exert an inhibitory influence on acetylcholine cells in the hippocampus (Robinson, Malthe-Sorenssen, Wood and Commissiong, 1979). Perhaps after an insult, a compensatory mechanism may result in this inhibition being reduced.

The results of the ChAT assays show that injected Al is able to enter the brain of the developing foetus and in some way affect the developmental timetable of the cholinergic system. This is perhaps not surprising when one considers the known immaturity of the rodent's blood-brain barrier during the foetal and perinatal period which creates the possibility of exposure of the foetal brain to agents within the mother's system. ChAT activity consistently increased in all brain regions studied between weeks 3 and 17 postpartum, then fell consistently during weeks 17-34. The levels of ChAT present in the oldest tissue samples between 34 and 44 weeks did not conform to such a predictable trend across brain regions or between experimental groups. Thus, the direction of change in ChAT levels was not always consistent in each brain region at each age, the greatest disparity occurring within the midbrain. This inconsistency

is difficult to explain but similar findings have been reported. Gulya, Rakonczay and Kasa (1990) reported decreases in the level of ChAT activity in the frontal cortex, parietal cortex, hippocampus and striatum of female Wistar rats exposed to $AlCl_3$ for either 5, 15 or 25 days. This decrease in activity not only varied from region to region but also showed inconsistent trends within the same region as a function of exposure time. For example, ChAT activity in the hippocampus, although consistently reduced compared to control levels, increased from 455±40 pmol/min/mg protein after 15 days of i.p. exposure to 507±30 pmol/min/mg protein after 25 days of exposure (Gulya, *et al.*, 1990).

The loss of ChAT activity in the various brain regions as a result of prenatal exposure to Al is complicated by the natural fall in ChAT activity and other markers of the cholinergic system with age. For example, Williams (1991) found age-related decreases of 15-30% in ChAT activity in the septum, hippocampus, frontal cortex and striatum of 24 month old Fisher 344 male rats compared to 4 month old counterparts. Gilad, Rabey, Tizabi and Gilad (1987) reported a reduction in choline uptake and acetylcholine release in 24 month old compared to 3 month old Wistar-Kyoto rats. Thus, there is a natural age-dependent degeneration of cholinergic neurons.

Animal studies involving surgical lesions of specific brain regions or damage induced by neurotoxins, have established the plasticity, both morphologically and biochemically, of the adult central nervous system to such injury (Cotman and Nieto-Sampedro, 1984). Steward and Messenheimer (1978) found an increase in the level of acetylcholine esterase, the enzyme responsible for the degradation of acetylcholine, 10 days after lesion of the entorhinal

cortex, the region from which the extrinsic input to the hippocampus arises, reflecting sprouting of the remaining septohippocampal fibers. Therefore, the lack of difference between control and treated groups at the oldest age in some brain regions may have resulted from the initiation of compensatory mechanisms which either increase the level of enzyme produced or enhance the sensitivity of the remaining neurons to the enzyme activity available, with the net result of little overall functional change.

Thus compensatory adaptations are initiated as a result of injury and aging. The experience of an insult during development may have initiated these responses earlier in the Al-treated animals so that modification of the neuronal circuitry was already underway by the time the stimulus for modifications induced by aging set in.

The disparity between the extensive effects of prenatal exposure to Al on ChAT levels and the relatively modest effects on behaviour has been reported elsewhere. Bartus, Flicker, Dean, Pontecorvo, Figueiredo, and Fisher (1985) found a profound effect of ibotenic acid (an excitotoxin) lesions of the nucleus basalis magnocellularis of male Sprague-Dawley rats on memory which gradually completely recovered despite continued cholinergic deficits. Interestingly, in agreement with the ChAT results reported here, these authors did not find a compensatory increase in ChAT levels with age. Further, Loesche and Steward (1977) correlated the recovery of performance in a T-maze with the reinnervation of the dentate gyrus following lesion of the entorhinal cortex by remaining neurons of the contralateral entorhinal cortex. Moreover, Gilad, *et al.*, (1987) have shown that loss of hippocampal pyramidal neurons is accompanied by an increase in muscarinic binding by remaining

neurons. Thus several compensatory mechanisms have to be exhausted before behavioural deficits are induced. Furthermore, Lippa, Pelham, Beer, Critchett, Dean and Bartus (1980) have suggested that memory impairment may not be detected until 20 months of age in rats. Although a decrease in latency to enter the shock compartment in a passive avoidance test was evident at 15 months of age, maximal effects were not reached until after 20 months (Lippa, *et al.*, 1980). Perhaps this represents a critical level before which the degree of loss of neurons or enzyme activity is balanced by continuing compensatory adaptations to such deficits. It is not until this critical point has been traversed that behavioural deficits are induced.

Overall the behavioural effects are not as consistent as the loss of ChAT activity. In terms of learning tests, Lochry, Hoberman and Christian (1985) have emphasised the importance of selecting an appropriate criterion to differentiate control and prenatally treated subjects. This disparity may have been reduced by incorporating further behavioural tests. For example, rats (no strain given) treated from postnatal day 2-3 to day 20-21 with lead acetate at a dose 81mg/kg, performed at comparable levels to controls on a one-way shuttle-avoidance test but learning deficits were seen in a two-way task (Sobotka, Brodie and Cook, 1975).

Finally, this disparity may also be explained in terms of information redundancy within the brain. Two and four exposures of rats (no strain given) to X-irradiation resulted in a 59 and 77% reduction respectively in granule cells of the dentate gyrus of the hippocampus compared to controls, but no differences in behavioural performances between exposed and control rats (Bayer, 1989). This

led Bayer (1989) to conclude that there is "*a cushion of neuronal redundancy in mammalian brains.*" Thus a certain degree of loss in, for example, cell number can be resisted before any behavioural changes are evident. This is interesting from a clinical point of view. It is known that the symptoms of Parkinson's disease are not manifested until the reduction in cell number within the substantia nigra is 80% relative to controls (Bayer, 1989).

CHAPTER 4

Effect of prenatal exposure to aluminium sulphate on ultrasonic calling in two inbred strains of mice.

4.1 INTRODUCTION

The experiments presented in this chapter and the next, describe in more detail aspects of behaviour which have proved to be sensitive to prenatal drug treatments.

The dialogue between a mother and her offspring involves delicate and highly complex interactions (Rosenblatt and Lehrman, 1963; Robinson and D'Udine, 1982). Although a number of studies have concentrated on the behaviour of the mother or the pups in this relationship, its intricacy, as pointed out by Robinson and D'Udine (1982), is probably reflected in the fact that both mother and infant contributions must be considered together rather than in isolation. Smith and Sales (1980) have emphasised the importance of this relationship, involving the continual exchange of stimuli between mother and young, in the successful shaping of the physical and behavioural development of the offspring.

It is probably due to their apparent immaturity that the contribution made by the infant to the mother-young relationship has been underestimated, although the newborn of altrical animals are quite adept physiologically and behaviourally. Nevertheless, it is difficult to define adequate measures of the behaviour of neonates which are both quantifiable and reliable, and which accurately reflect changes resulting from alterations to environmental variables. However, infant rodents do possess the ability to emit calls within the ultrasonic range, which are given in response to a variety of

stimuli. As pointed out by Zbinden (1981), and confirmed by Cuomo, De Salvia, Maselli, Santo and Cagiano (1987), this response may function as a reliable indicator of subtle behavioural effects caused by prenatal or postnatal drug treatments.

Anderson (1954) was the first to record ultrasonic emissions from adult laboratory rats placed alone in their cages. In 1956 Zippelius and Schleidt (cited in Noirot, 1966) described the production of ultrasounds by young from different rodent species. The production of ultrasounds by a number of rodent species is now well established (e.g. Sewell, 1967; Okon, 1972; Sales and Smith, 1978; Elwood and McCauley, 1983). Furthermore, since these initial descriptions research has concentrated on factors affecting ultrasonic output, analysis of the physical characteristics and the possible function of these calls.

For the purposes of this chapter I will concentrate on infant ultrasonic calling although several circumstances exist during which adult animals will emit similar calls and these are discussed briefly.

4.1.1 Adult ultrasonic calling

Sewell (1967) and Sales (1972a; 1972b) described the emission of ultrasounds when an adult male rat was introduced into the cage of a conspecific, of a lactating or pregnant female and also during episodes of mounting. She concluded that these calls were important in the social life of adult rodents. Additionally, Okon (1972) noted that nursing mice produced ultrasounds similar to those emitted by infants. Further, Lewis and Schriefer (1982) found that pregnant Sprague-Dawley rats exhibited a higher rate of calling than

controls (non-pregnant rats) on the day before gestation. Thus, in the areas of aggressive and sexual behaviour, ultrasounds are important in the adult animal.

4.1.2 Ultrasonic calling by neonates

Since its initial description the production of ultrasonic emissions by a number of neonatal rodent species as a form of communication between young and mother has been well characterised. Several circumstances have now been found which evoke calling in infant rodents. Calls may be elicited as a result of gentle handling (Okon, 1970b; Bell, Nitschke and Zachman, 1972), during isolation (Noirot, 1968; Robinson and D'Udine, 1982), under cold stress (Hart and King, 1966; Noirot, 1968; Okon, 1970a; Okon, 1972;), or from exposure to unusual tactile (Okon, 1970b) or olfactory stimuli (Oswalt and Meier, 1975; D'Amato and Cabib, 1987).

As pups develop from birth characteristics of their ultrasonic vocalisations change. Noirot (1966, 1968) found that isolated albino mouse or rat pups show a large increase in calling on day 4 postpartum, corresponding to the opening of the ears. There is a decrease in calling on day 13 which coincides with eye opening. Noirot and Pye (1969) also found regular changes in ultrasonic emissions with successive days.

These ontogenetic changes in ultrasonic output have been related to the development of thermoregulation. With a decrease in ambient temperature the rate of calling characteristically increases with age to a peak and subsequently falls until it eventually ceases

completely. The exact time course of these events is dependent on the species and strain of the subjects. Lagerspetz (1962) simultaneously recorded ultrasonic emissions and body temperatures from mice of the inbred Swiss albino strain at various ambient temperatures. He found that during the first few days of life rodent pups are entirely poikilothermic; as the ambient temperature drops so does the pup's internal temperature. At this age rodent pups are extremely resistent to cold; a decline in temperature causes the pup to become comatosed and hence it ceases to produce calls. As homiothermy develops the pup reacts to a drop in temperature by intensive calling. Okon (1970a) found this phase to be around days 6-7 postpartum for the Swiss albino mouse. At a later stage, when the development of thermoregulation is complete, a reduction in ambient temperature is not such a strong stimulus and calling is again reduced.

Although Noirot (1966) related the cessation of calling to the stage of eye opening, Okon (1970b, 1972) has elicited calls from pups after this by handling. It is more likely that alterations in calling in response to a reduction in ambient temperature are related to the development of homiothermy and will cease when this has been achieved.

Young rodent pups have been found to respond to tactile stimuli by calling although the pattern of calling is not the same as that evoked by temperature reduction. Okon (1970b) found that, in response to handling, albino mouse pups have the greatest rate of calling during days 1-3 postpartum after which the rate declines until day 14 when no further calls are elicited. This profile of calling is also highly dependent on species and strain. Even using similar

methods to elicit calling, different researchers have reported varied profiles of calling. For example, Noirot and Pye (1969) described a high rate of calling on Pd1 in response to chilling which was followed by a decrease over the next 6-7 days, the reduction in temperature compared to the maternal nest being the stimuli for calling. On the other hand, using the same mouse strain, Okon (1970a) found that pups exhibited a low response until day 6 after which there was a large increase in the rate and intensity of calling.

In most experiments there is an inevitable confounding of stimuli on the pups. In order to isolate a pup one must handle it to remove it from the nest which in turn lowers the pup's temperature and so on. Noirot and Pye (1969) reported deliberately having gently handled the pups to elicit calls during recording, thus complicating the interpretation of their results of calling elicited by temperature reduction whereas Okon's (1970a) pups, once removed from the nest, were exposed solely to a decrease in ambient temperature.

Despite the difficulties in isolating stimuli which elicit calling, two different patterns of ultrasonic calling have been described; one produced in response to handling and one resulting from being isolated. These are thought to correspond with the different communicative functions of calling. Ultrasounds elicited by handling are thought to inhibit the mother's continuing rough handling of the pup during retrieval or rough grooming (Noirot, 1966; Sewell, 1970). Those resulting from isolation elicit maternal care from the mother (Zippelius and Schleidt, 1956 cited in Noirot, 1966; Beach and Jaynes, 1956; Sewell, 1970; Allin and Banks, 1972). Noirot (1966) found that mothers would actively search for isolated pups and retrieve them in response to these calls. Moreover,

Reisbick, Rosenblatt and Mayer (1975) have shown that fostering younger litters to lactating females prolongs the time over which these females respond maternally. Allin and Banks (1972), using playback of recorded pup calls, found that ultrasounds elicited the response of head orientation from males, virgin females and lactating females but it was only the latter group which actually left the nest and exhibited searching behaviour. They concluded that ultrasounds were important in providing information on the direction of the scattered pup. Similarly, Noirot (1974) found that virgin females would alter their behaviour on exposure to an inaccessible litter exposed to a reduced temperature. Smotherman, Bell, Starze, Elias and Zachman (1974) reported that both pup odour and ultrasonic calling were the strongest stimuli for accurate and rapid retrieval of a lost pup.

Characteristics of ultrasonic vocalisations have not only been found to be quite different between species but also between subspecies. For example, Hart and King (1966) found that under conditions of cold stress young from two species of deermice (*Peromyscus maniculatus bairdii* and *P. m. gracilis*) differed significantly in all of the physical measures recorded. Wistar rat pups emit few calls in response to handling until days 4-5 postpartum when the rate of calling increases until days 14-15. On the other hand, bank vole pups (*Clethrionomys*) appear to be completely insensitive to calling evoked by tactile stimuli (Okon, 1972).

There are clear examples of genetic differences in calling from comparisons of pups from various strains within the same species. Bell, Nitschke and Zachman (1972) recorded ultrasounds from three different strains of mice (C57BL/6J, BALBc/J and

C3H/He) upon removal from the nest every third day from postnatal day 3 to 18. Characteristically the ultrasonic emissions differed with age but further the timing of the peak frequency and duration of calling differed significantly between strains. Similarly, Robinson and D'Udine (1982) measured the effects of isolation alone on calling in three inbred lines (BALB/c, SEC and C57BL/6J). Each pup was placed on a heated blanket and ultrasonic output was recorded over five minutes. BALB/c and SEC mice produced significantly more calls on each day and for a longer period than pups of the same age belonging to the C57 strain.

Relatively few studies have investigated the effects of prenatal drug treatment on the maternal-infant relationship. To my knowledge even fewer have recorded changes in ultrasonic output as a result of such exposure, despite the reliable and stable pattern of ultrasonic calling by rodent neonates. This is perhaps more surprising in the light of research which has shown that alterations in maternal care can affect the subsequent behaviour of the offspring (e.g. Deitchman, Kapusinski and Burkholder, 1977) and that pup stimuli are responsible for eliciting adequate maternal responsiveness. Within the studies which have incorporated this technique, both increases and decreases in calling have been reported. Wistar rat pups treated chronically with haloperidol, a dopamine antagonist, during the early postnatal period (days 2 to 16) exhibited a reduced rate and frequency of calling when they were isolated (Cagiano, Sales, Renna, Racagni and Cuomo, 1986). Conversely, Adams (1982) found that Sprague-Dawley rats treated prenatally with vitamin A palmitate (80,000 IU/kg), a known

behavioural teratogen, called at a significantly higher rate than controls.

It is clear from chapter 3 that there is a highly significant effect of the treatment received by a pup's foster mother during gestation on aspects of a pup's physical and behavioural development. The pattern of ultrasonic emissions has been recorded in this experiment in an attempt to investigate a further, and more detailed aspect of the mother/pup inter-relationship.

It must be noted that removing a pup from the nest and placing it alone at a lower temperature is potentially stressful. The calls produced in this experiment have probably resulted from a reaction to a compound of changes, the drop in temperature, being isolated from the normal tactile and olfactory stimuli of the mother and littermates, and maybe even from hunger as pups suckle continuously during the early postnatal period. However, this experimental design does not aim to investigate the factors which cause calls to be emitted but whether pups in the four experimental groups have the same pattern of calling, and what its effect is upon their mothers.

As reported in section **3.9**, several attempts were made to produce enough C57 pups to enable cross-fostering between litters but C57 females failed to continue to term probably because few were actually plugged rather than as a direct result of exposure to Al. Thus, the results for C57 ultrasonic calling presented in this chapter are infact the results from the offspring of the mothers observed for maternal behaviour in chapter 5. For this reason only pup weights and ultrasonic calling were measured so as not to cause further disturbance to these mothers.

Some of the work described in this chapter has been accepted for publication (Rankin and Manning, in press, see Appendix C).

4.2 METHOD

4.2.1 Treatment

Plugged CBA and C57 females were randomly divided into two groups which received either i.p. injections of $Al_2(SO_4)_3$ or the equivalent volume of saline at the same pH, on gestation days 10 to 13.

4.2.2 Procedure

Due to the differences in the pattern of ultrasonic calling of the two strains, recording took place over 6 days (on Pd3,4,5,6,9,12) for the CBA strain and on 4 days (Pd3,4,6,8) for C57 pups. These days were chosen on the basis of pilot studies and from previous work which typically showed that few calls are emitted at birth by either strain and C57 pups have ceased calling by Pd10 and CBA pups by Pd12-13.

The litter was removed from the home cage and placed in a glass crystallising dish lined with nesting material (dish 1), which had previously been warmed to a comparable temperature as the maternal nest (34-36^{o}C) with a lamp (25W). Each pup was then removed individually from dish 1 and placed in a second dish (dish 2) which was far cooler (24-26^{o}C) and acted as a mild thermal

stressor. Ultrasounds were recorded using a QMC Mini-2 Bat detector set to receive a frequency band of 70-80 kHz (see Noirot, 1966; Okon, 1970a; Robinson and D'Udine, 1982), and mounted 10 cm above dish 2. The detector is equipped with a microphone and headphones. The number of calls emitted was counted using a hand tally and noted at the end of each minute for a period of 5 minutes. Scoring of ultrasounds was begun 1 minute after placing the pup in dish 2 to allow any immediate response to handling to die down. The same type of nesting material as used for the maternal nest was placed in dish 1 to reduce sound emissions due to unusual tactile stimuli (Noirot and Pye, 1969; Okon, 1970b) although odours from the mother and littermates were not present.

Recording over five minutes was thought to be sufficient to even out stort-term fluctuations in calling output. It is not uncommon for mouse pups from certain strains to produce calls at a rate greater than 250/minute (Noirot and Pye, 1969). In these instances the method of recording may have underestimated the number of calls produced, although this will have been the case across all experimental groups. After recording the pup was replaced into dish 1 and the procedure repeated for each pup in the litter. Individual pups were not returned to the home cage until all pups in the litter had been tested. The order of testing of both litters and individual pups was randomised on each test day.

4.2.3 Maternal Retrieval

This was carried out for CBA mothers only. On completion of recording of ultrasonic calling the four pups were replaced into

the home cage at the opposite end from the maternal nest. The time taken to return one of the pups to the nest was recorded.

4.2.4 Pup measures

Body weight was recorded on every third day from Pd3 to weaning at Pd21 for CBA and C57 pups. On these days CBA pups underwent the modified version of the Fox tests for sensory-motor coordination (Fox, 1965), as reported in the general methods chapter (section *2.2.1*).

4.3 RESULTS

The main ANOVA tables and median + lower and upper interquartile ranges are given in Appendix D.

4.3.1 Breeding Performance

Prenatal exposure to $Al_2(SO_4)_3$ during gestation days 10-13 had no effect on breeding performance in either strain. The mean gestation length for control CBA mothers (n=10) was 19.9($\pm$0.23) days and 20.0($\pm$0.37) days for those CBA females exposed to Al (n=9). C57 Al-exposed females (n=11) had a slightly shorter mean gestation length ($\bar{x}$=20.73($\pm$0.56) days) compared to C57 controls (n=8, $\bar{x}$=21.13($\pm$0.85) days), although this difference was not significant. Pup mortality occurred in both control and treated groups in the CBA and C57 strains at birth, as was confirmed by the presence of blood and from the body weight records taken during

pregnancy. Although control females from both strains were found to have larger mean litter sizes (CBA $\bar{x}$=6.0(±0.71); C57 $\bar{x}$=6.75(±0.37)) compared to Al-treated females (CBA $\bar{x}$=4.8(±0.8); C57 $\bar{x}$=5.82(±0.48)), these differences were not significant.

4.3.2 Maternal weight

There was no significant effect of prenatal treatment on CBA maternal weight during gestation, although there was a significant day and prenatal treatment interaction ($F(3,24)=10.66$ $p=0.004$). The overall weight gained by CBA treated mothers was significantly less during gestation days 10-18 than CBA control mothers (control $\bar{x}$=8.69(±0.7)g, treated $\bar{x}$=5.65(±0.81)g, $t(16)= 2.84$ $p<0.02$). C57 maternal weight gain was largely unaffected by gestational exposure to $Al_2(SO_4)_3$, although the total mean weight gain for control females (n=8 $\bar{x}$=10.18(±1.11)g) was slightly greater than Al-exposed females (n=11 $\bar{x}$=8.98(±0.66)g).

CBA maternal weights were largely unaffected by treatment during the preweaning period, except for a marginally significant interaction between day and prenatal treatment, control CBA mothers being slightly heavier during the latter days of the preweaning period. C57 females gained weight at a comparable rate.

4.3.3 Pup weight

A total of 46 control and 25 Al-treated CBA pups were present at birth. There was a highly significant difference in the mean birth weights of control and treated CBA pups (control

$\bar{x}$=1.35(±0.02)g, treated $\bar{x}$=1.19(±0.03)g, $t(37)$= 3.95 p=0.0003), pups exposed to aluminium being 12% lighter. After cross-fostering there was a total of 40 pups, 2 pups were neglected by their foster mothers and one pup was not included in the analysis as it was a runt, leaving a total of 37. The remaining pups were distributed within the experimental groups as follows:- **Cc** n=10, **Ct** n=10, **Tt** n=9 and **Tc** n=8.

Within the C57 strain, 49 control and 57 treated pups were present at birth. As with CBA pups, C57 controls weighed more at birth than treated offspring (control $\bar{x}$=1.5(±0.02)g; treated $\bar{x}$=1.36(±0.02)g, $t(103)$= -5.37 p<0.0001). Pup sample sizes in the C57 strain were:- **Cc** n=13, **Ct** n=16, **Tt** n=16 and **Tc** n=11.

As in experiment 1 of chapter 3, there was no effect of sex on CBA pup body weights up to weaning. There was a highly significant effect of prenatal treatment (F(1,29)=30.74 p<0.0001) and foster mother treatment (F(1,29)=30.34 p<0.0001) on pup weight during the preweaning period. As can be seen from Fig. 4.1, **Cc** pups were found to be the heaviest group and **Tt** pups weighed the least, a difference of 20% by Pd21. Again, as in experiment 1, the experimental groups run parallel with their foster mother's treatment; those pups fostered to control mothers weighed more than pups reared by mothers who had been exposed to Al. For example, by weaning **Ct** pups weighed 11% less than their control counterparts (**Cc**) and similarly **Tt** pups were 14% lighter than **Tc** pups. There were also significant interactions between day and prenatal treatment (F(6,174)=4.61 p<0.004), and day and foster mother treatment (F(6,174)=8.88 p<0.0001).

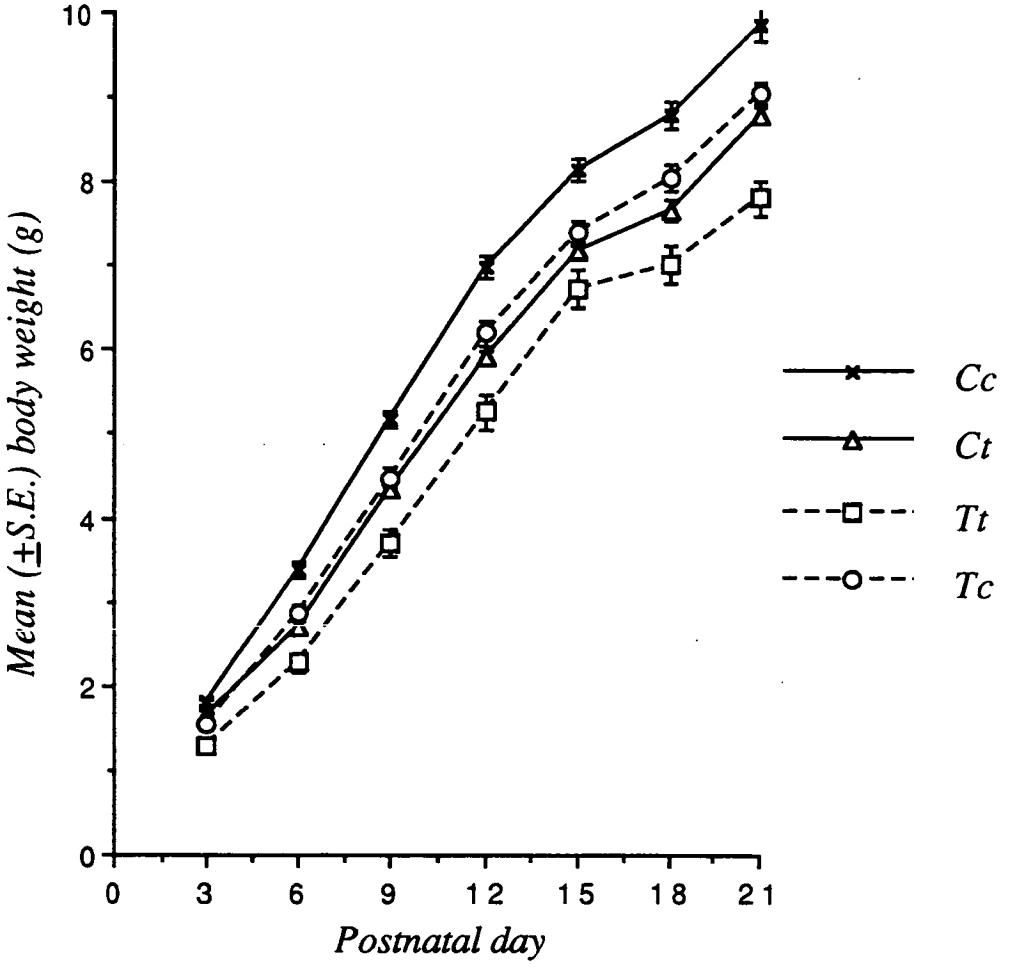


Fig. 4.1: Body weights of CBA pups whose mothers received intraperitoneal injections of either aluminium sulphate (200mg/kg) or saline during Gd10-13.

In the C57 strain, male and female pups were separated for analysis due to the influence of sex on body weight ($F(1,47)=5.83$ $p<0.02$). Figs. 4.2a and b summarise male and female pup body weight change during the preweaning period. One can see that **Cc** pups weighed the most and **Ct** and **Tt** pups, whose lines are almost overlapping, the least. This difference was most pronounced by Pd12 when **Cc** pups were 10% heavier than **Ct** or **Tt** pups. There was a significant overall effect of foster mother treatment on C57 male pup weights ($F(1,26)=4.83$ $p<0.04$), although the day and foster mother treatment interaction was only marginally significant. C57 pups fostered to treated mothers had lower body weights than pups fostered to control mothers, the extent of this difference declined with age.

The divergence in body weight between C57 female pups was greater than for C57 males and increased with age (see Fig. 4.2b). As with C57 males, there was a significant effect of foster mother treatment ($F(1,21)=7.94$ $p=0.01$) and an interactive effect of day and foster mother treatment ($F(6,126)=5.25$ $p=0.01$) on female body weight, females raised by treated mothers weighed less than those reared by control females, a difference that increased with time.

Thus, in the two strains the lower weight of treated pups at birth continued throughout the postnatal period for those pups fostered to treated mothers only.

Fig. 4.2a

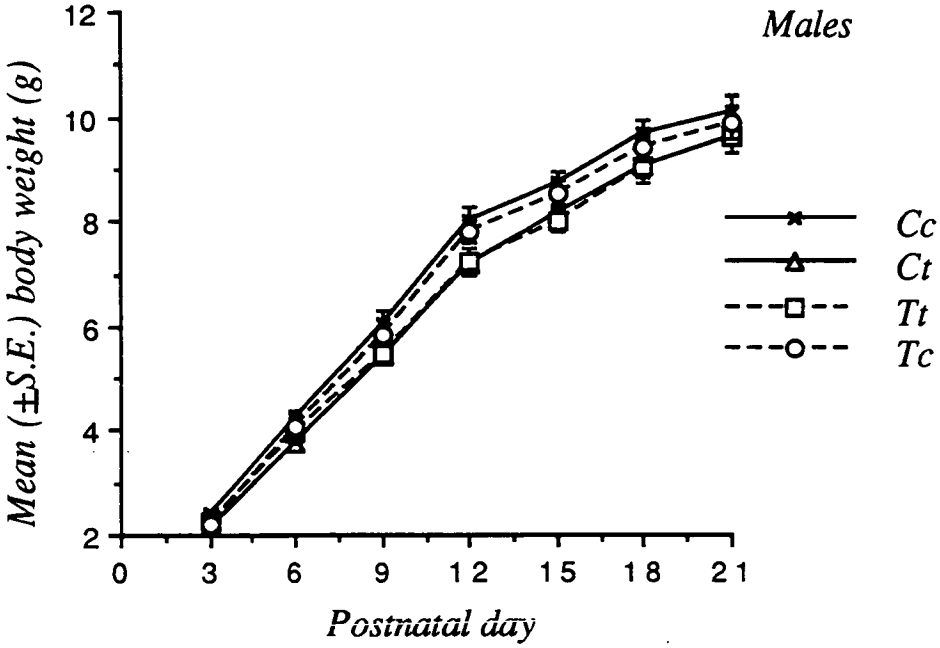


Fig. 4.2b

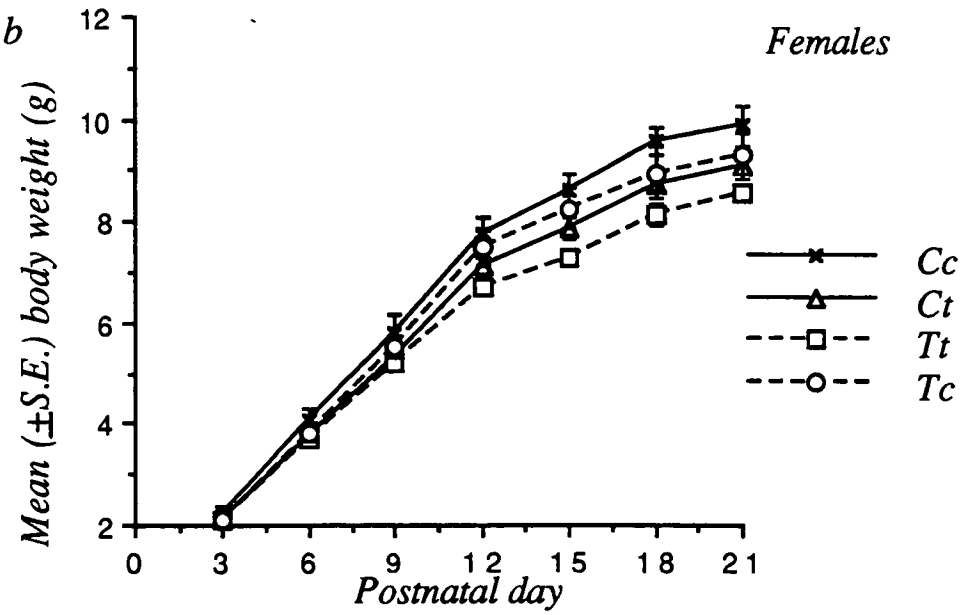


Fig. 4.2: Body weights of C57BL/6J pups whose mothers received intraperitoneal injections of either aluminium sulphate (200mg/kg) or saline during Gd10-13.

4.3.4 Fox tests

Chi-square analysis of the 7 Fox tests employed revealed a highly significant difference between CBA pups in *forelimb grasping* on Pd12 ($X^2(3)=20.401$ $p<0.001$), with only 2 out of 9 **Tt** pups having reached an adult response, and in *screen climbing* on Pd15 ($X^2(3)=11.16$ $0.05>p<0.02$). Performance in the other tests was not found to differ significantly between the groups and the differences in the two tests above were not correlated with weight.

There is no data available for performance of C57 pups in the Fox tests.

4.3.5 Ultrasonic calling

Figs. 4.3a and b summarise the changes in the total median number of ultrasounds emitted on each test day by the four experimental groups for CBA and C57 pups respectively. In the case of CBA pups, calls were most vigorously emitted during the earliest postnatal days, reaching a peak which then steadily declined so that by day 12 very little calling was evoked by the drop in temperature, as the development of adequate homiothermy was finally achieved. It is not possible to say when calling ceased completely as no further measurements were taken after Pd12. Unlike pup weights there was no influence of sex on C57 calling. C57 pups did not exhibit as clear a pattern of calling with age on exposure to a drop in temperature.

The most striking observation from the CBA data is that, throughout the test period, the total number of calls emitted was dramatically reduced in those pups exposed to Al *in utero*. This

Fig. 4.3a

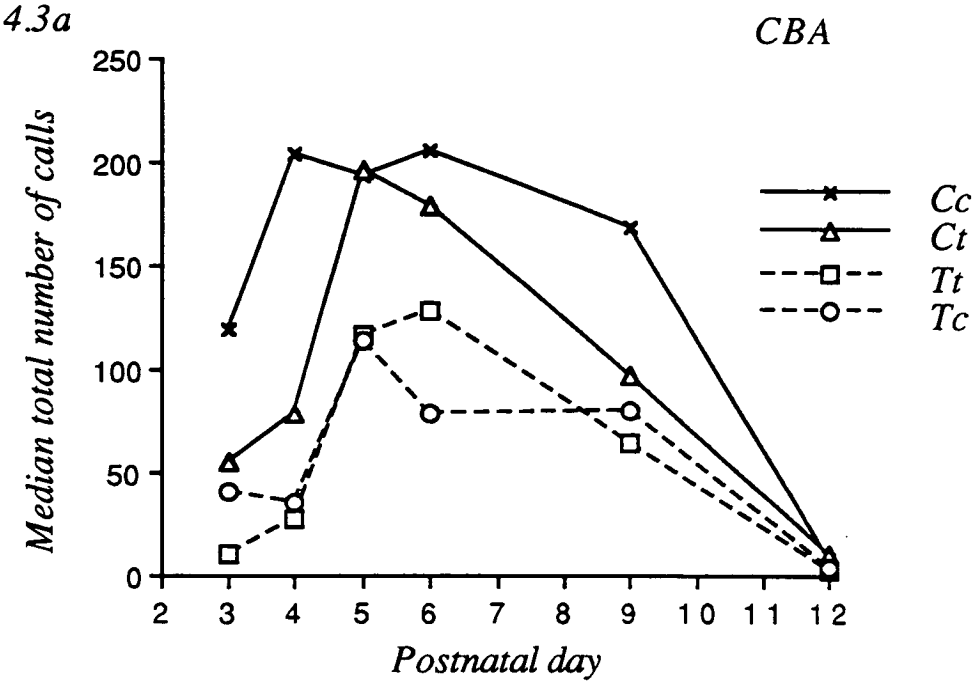


Fig. 4.3b

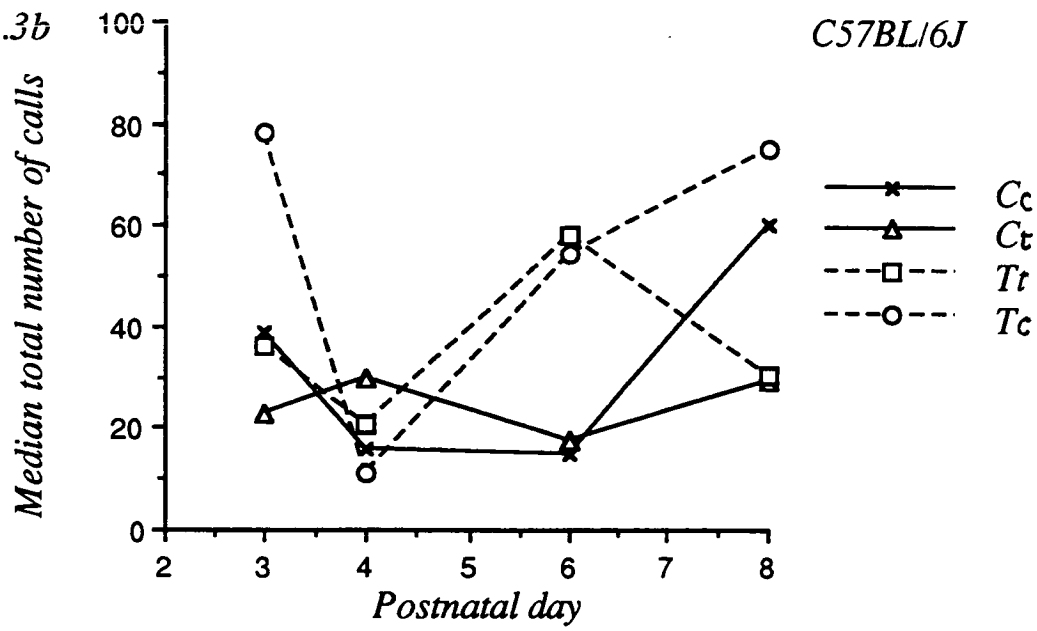


Fig. 4.3: The total median number of ultrasonic calls emitted by (a) CBA and (b) C57BL/6J pups in a five minute test.

difference was especially pronounced on Pd3 ($H(3)=12.79$ $p=0.005$), Pd4 ($H(3)=20.95$, $p=0.0001$) but only reached marginal significance on Pd6. The rate of calling of **Cc** pups increased to a peak on Pd4 (204.5 calls/min) and then slowly declined to a median of less than 2 calls/minute by Pd12. **Ct** and **Tc** pups produced their greatest median number of calls on Pd5 (196.5 and 114.5 calls/min respectively) and **Tt** pups on Pd6 (128 calls/min). Although control pups from both groups emitted more total calls than treated pups, **Ct** pups had a considerably lower initial rate of calling on Pd3 than **Cc** pups. Their rate of output reached comparable levels to that of **Cc** pups only by Pd5. The decline in calling was also more dramatic for this group. Thus, being raised by a treated mother dampened the rate at which these pups called. The multiple comparisons option revealed that on Pd3 and 4 **Cc** pups emitted significantly more calls than the **Tt** group (at the $p<0.05$ level) and on day 4 groups **Cc** and **Tc** differed significantly. No other comparisons were significant.

In summary, prenatal Al exposure caused two effects; it resulted in a reduction in the rate of calling and altered the day on which pup calls reached a maximum.

Any disruption to the ultrasonic calling of C57 pups by *in utero* exposure to Al is not as clearly apparent. **Cc** pups decreased their total median number of calls between days 3 and 4, then increased by Pd8. **Tc** pups displayed a similar pattern of calling as that of **Cc** pups although their initial median total was greater. **Ct** pups exhibited a fairly constant low level of calling of a median of 30 calls/5 minutes. Pups in the **Tt** group had a more irregular pattern; the total median number of calls decreased between Pd3 and 4, increased to Pd6 and then fell again. Kruskal-Wallis ANOVA

revealed a marginally significant difference between experimental groups on Pd8 only, pups fostered to control mothers calling more than those reared by treated mothers.

4.3.6 Maternal retrieval

CBA control mothers had a reduced latency to retrieve the first pup back to the nest on Pd3 (control n=5 median=24.0s, treated n=5 median=54.0s) but this was not found to be significant. As pups grew older both groups tended to reduce their latency to contact the pup. Maternal retrieval was not tested on Pd12 as pups were capable of homing to the nest before the mother could pick them up.

Maternal retrieval was not recorded for C57 mothers so as not to disturb them during the maternal behaviour experiment undertaken in chapter 5.

4.4 DISCUSSION

Some of the trends recorded in this experiment for the CBA strain are in agreement with those previously described in chapter 3. In that experiment the treatment received by a pup's foster mother during gestation was found to have a marked effect on pup body weight up to weaning. Al-treated pups also exhibited developmental delays in several of the Fox tests and activity levels at weaning were found to be both sex and prenatal treatment dependent. By contrast, the results of the experiment reported in this chapter show that pup weights were not only influenced by foster mother treatment but additionally by direct exposure to Al prenatally. Similar maturational

delays were present in both experiments. Additionally, the implication of the results on CBA ultrasonic calling is that, unlike pup weights, the production of ultrasounds is largely unaffected by foster mother treatment but is strikingly reduced by exposure to $Al_2(SO_4)_3$ *in utero*. On the other hand, any alteration to ultrasonic calling of C57 pups is harder to discern because of the low response level of this strain to a reduction in temperature, although there is a slight suggestion of an influence of maternal treatment. The lack of differences between the C57 experimental groups probably results from a floor effect; the baseline level of calling is so low that any disruption is unlikely to be evident.

The strain differences in ultrasonic calling are quite obvious from figures 4.3a and b, in particular the greatly diminished number of calls produced by C57 pups which never exceeded a median total of 80 calls in a five minute period. Robinson and D'Udine (1982) found that C57 pups never produced calls at a median rate greater than 30 calls in 5 minutes, with a peak between days 2-4 postpartum and a cessation of calling by Pd10. In this experiment, peak calling for **Cc** pups occurred on Pd8, Pd4 for **Ct**, Pd6 for **Tt** pups and Pd3 for the **Tc** group.

A limitation in the method of recording is that it does not provide information on the mode of calling i.e. whether one group called more frequently in bursts whilst the other called continuously. However, examination of the data minute by minute revealed that treated pups exhibited both fewer bursts and a lower number of calls within these bursts compared to controls.

It was suggested in the introduction to this chapter that recording of ultrasonic calling might provide a reliable measure of

toxicity following exposure to drugs or other toxicants. In the light of the results presented herein, it must be noted that the strain of the subject is an important consideration and the reliability of this measure will be greater for those strains with a high level of calling. Thus, the following discussion refers to the alterations in the calling pattern of CBA pups.

It is not possible to determine with confidence whether the disparity in the production of ultrasonic calls by CBA pups was due to effects of exposure to Al on the vocalisation system *per se*, or on its rate of maturation. It is unlikely that this difference can be explained in terms of a damaged vocalisation apparatus as pups from all the experimental groups did exhibit the ability to call. The peak calling occurred a day later for treated CBA pups than controls. It would certainly seem possible that the mechanisms of sound production matured more rapidly in control pups. Alternatively, the calls elicited by the treated pups may have been weaker or of lower intensity than those produced by control pups. Although unlikely, calls from treated pups may have had differing physical characteristics outside the range of the detector. Thus, prenatal treatment may have in some way shifted the acoustic parameters of the calls from CBA pups.

The results of the sensory-motor tests, in which CBA pups exposed to Al prenatally were, in some respects, slower to mature than control pups, gives some support to this idea that the differential profiles of calling for each experimental group may have resulted from a delay in the maturation of the mechanisms responsible for ultrasonic vocalisations.

However, other possible explanations must be considered. The differential calling profiles may be related to changes in the pup's detection of, or response to, chilling. As discussed in the introduction to this chapter, several investigators have established a correlation between the emission of ultrasonic calls and the development of homiothermy (Lagerspetz, 1962; Sewell, 1968; Okon 1970a; Allin and Banks, 1971). The typical age profile for calling when exposed to a decreased ambient temperature is one of few calls immediately after birth, coinciding with the period of poikilothermy, followed by a greater intensity of calling corresponding to the initial development of thermoregulatory ability and finally a subsequent decrease in the rate of calling as thermoregulation is achieved. This is the calling profile followed by **Cc** pups, whereas treated pups did not exhibit the characteristic changes with age. Perhaps exposure to Al had in some way prolonged the period of poikilothermy which prevented them from calling. To eliminate this possibility would require investigations into the processes underlying temperature regulation. However, to help focus the effects of Al on the vocalisation system it would be interesting to record calling ouput following a different method of induction e.g. by tactile or olfactory stimulation.

Prenatal exposure to $Al_2(SO_4)_3$ did not dramatically alter the pattern of the distribution of calls over the 12 postpartum days but significantly reduced the number of calls produced. Thus, *in utero* Al dampened the call profile of the treated pups. This experiment aimed at assessing the alteration in infant ultrasonic emissions as a measure of their responsiveness to a mildly stressful situation. If ultrasonic calling functions to elicit maternal responses,

as has been proposed by several workers (Noirot, 1972; Sewell, 1970) and alterations in maternal behaviour are known to affect infant development (Laviola, Sedowofia, Innes, Clayton and Manning 1990), it would seem pertinent to establish what effect differences in ultrasonic output are exerting on maternal behaviour (see chapter 5).

Such differences in ultrasonic calling may result in treated pups being less than optimal stimuli for eliciting maternal care. Alteration in the pattern of maternal care might explain why **Ct** pups, who were found to emit a similar profile of calls although at a lower level compared to their **Cc** counterparts, had body weights notably reduced to that of **Cc** pups. Similarly, **Tc** and **Tt** pups had comparable profiles of calling but **Tc** pups were of greater body weight. In the case of **Tt** pups, the diminished number of calls may have affected the ability of the treated mother to provide adequate maternal responses even in the presence of calls from control pups. **Tc** pups had a similiar reduced profile of calls to that of **Tt** pups, the difference being that these treated pups were raised by control mothers. These mothers may have been in finer tune to their pups so that even a diminution in the number of calls was not sufficient to alter maternal responsiveness. However, despite the altered level and pattern of calling in treated pups, these results do not reveal any change to the mother's responses although maternal retrieval was the only maternal measure undertaken in this experiment.

As discussed in the Introduction, ultrasounds are thought to evoke searching and retrieval of lost pups by the mother to the security of the nest. Having pups which exhibit weak or low

numbers of calls would have serious repercussions in terms of survival of the litter.

It was suggested in chapter 3 that the body weight differences between CBA pups may be accounted for in terms of alterations in the output of ultrasonic calls by pups fostered to treated mothers. However, in the light of the ultrasonic data presented here, this no longer seems to provide an adequate explanation. If this had been the case CBA pups in the **Tc** treatment group, which exhibited a low rate of calling, should have had far lower body weights than controls but they clearly do not. Further, the body weights of male and female C57 pups showed similar impairments to that found in the CBA strain, namely fostering pups to mothers exposed to Al during gestation resulted in a diminished body weight. As little difference was found in C57 pup calling it is unlikely that alterations in ultrasonic calling contributed greatly to this reduction in body weight.

Terkel, Damassa and Sawyer (1979) claim that ultrasonic calling stimulates the release of prolactin from the mother's anterior pituitary (although Stern, Thomas, Rabii and Barfield (1984) do not confirm this). The primary stimulus for its secretion is probably suckling although several other features of the pup have been found to increase its release. If ultrasounds are indeed one such feature then the increase in sound emissions by **Cc** pups could have the effect of augmenting the nourishment available for them, which has obvious consequences for growth.

Results from the maternal retrieval tests showed that treatment of the young did not reduce their effectiveness as retrieving stimuli. This is perhaps not surprising for three reasons.

Firstly, although the procedure used to test maternal retrieval was a standard design employed by most studies, it may not have discriminated between experimental groups as the distance over which the females had to travel was small. Secondly, some mothers were seen to be having difficulty seizing a pup as it was moving especially during the later test days. During these episodes the mother would wander back and forth to the nest and eventually ended up dragging the pup to it. Finally, Beach and Jaynes (1956) have described a number of stimuli to which mother rats respond when retrieving their pups. For example, they showed that if the olfactory characteristics of a pup are altered (by covering it with perfume) the pup is still eventually retrieved although control pups are retrieved preferentially. Thus, even if Al affected one aspect of the pup's stimulus quality other sensory modalities would remain to evoke a normal reaction.

The exact mechanisms underlying the regulation of ultrasonic calling are not known and several systems including endogenous opiates (Cuomo, Cagiano, De Saliva, Restani, Galimberti, Racagni and Galli, 1988) the dopaminergic (Cagiano, *et al.*, 1986) and the benzodiazepine receptor complex (Insel, Hill and Mayor, 1986) have been implicated. However, Brudzynski and Bihari (1990) have suggested the involvement of the cholinergic system in their production. In chapter 3 (see section *3.4.11*) a reduction in ChAT activity was found in brains of mice exposed to Al *in utero*. That such treatment led to a reduction in calling is at least consistent with Brudzynski and Bihari's (1990) hypothesis. As there is only direct evidence for the cholinergic system the involvement of other neurotransmitter systems cannot be ruled out.

Finally, the results reported in this chapter certainly suggest that ultrasound recording should be included in any battery of tests aimed at assessing possible behavioural teratogenicity.

CHAPTER 5

Effect of gestational exposure to aluminium sulphate on the maternal behaviour of two inbred strains of mice.

5.1 INTRODUCTION

As discussed in the introduction to chapter 4, the mother-infant relationship involves the active participation of both members. Since prenatal exposure to Al reduced calling output in treated pups and also delayed the onset of peak calling, the observations undertaken in this chapter aimed at assessing the effect of gestational exposure to Al on the mother.

Females from a number of rodent species display a characteristic sequence of behaviours towards their young which is collectively referred to as maternal behaviour (Rosenblatt and Lehrman, 1963; Noirot, 1972). These behaviours are thought to be initiated by the hormonal changes which occur at the time of parturition, namely an elevation in the amount of circulating oestradiol which follows a decline in the level of progesterone (Rosenblatt and Siegel, 1983). However, maintenance of maternal care depends not only on hormonal influences but also on the efficiency of the bidirectional exchange of a number of sensory cues between the mother and her offspring, both playing an important active role. For example, as discussed in the previous chapter, ultrasonic vocalisations emitted by pups play an important communicative role in this relationship, specifically as cues to direct the mother to a strayed pup (Noirot, 1972). Further, olfactory stimuli are important in pup recognition, as evidenced from lesions of the noradrenergic projection to the olfactory bulbs in nulliparous

BALB/c females before parturition, which resulted in cannibalistic behaviour (Dickinson and Keverne, 1988).

More specifically, maternal behaviour is sensitive to subtle stimulus changes which can alter this behaviour and in turn may have long-term effects on the development of the offspring. For example, it has been suggested that the enhancing effects of early handling procedures on pup development may be explained in terms of changes in maternal behaviour; several studies have described increased licking behaviour by mothers following handling of their young (Lee and Williams, 1974; Priestnall, 1983).

Postnatal social influences have also been found to modify maternal behaviour. Alleva, Caprioli and Laviola (1989) demonstrated the influence of the sex composition of a litter on maternal behaviour; litters comprised solely of male offspring received more pup-directed maternal activities than litters made up of all females or with equal numbers of male and female offspring.

Recently, studies which involve the investigation of the effects of administration of drugs or toxicants during pregnancy on offspring development, have examined the effect of such treatments on the expression and pattern of maternal behaviour. Alcohol-exposed CFW females were found to exhibit an increase in the frequency of exploratory behaviour one day after birth (Ewart and Cutler, 1979). Interestingly, Hard, Musi, Dahlgren, Engel, Larsson, Liljequist and Lindh (1985) found that female Wistar rats, exposed *in utero* to ethanol (16% ethanol solution), took longer to retrieve their pups and built nests of a lower depth than controls.

Enhancements of maternal behaviour have also been reported following prenatal treatments. Outbred female mice

exhibited an increased duration of time involved in nursing and an increased frequency and duration of Social Investigation of their young on Pd1 following exposure to phenobarbitone (PhB) in their drinking water (120-190mg/kg daily) throughout gestation and lactation (Chapman and Cutler, 1983). At Pd21 PhB-treated females were still actively engaged in nursing behaviour (a mean duration of 61s for treated females compared to a mean of 0.4s for the control group). Furthermore, Laviola, Sedowofia, Innes, Clayton and Manning (1990) have demonstrated strain-specific differences in the influence of prenatal exposure to PhB (60mg/kg by i.p. injections during days 10-16 of gestation) on maternal behaviour. CBA treated females continued to nurse their offspring for longer than controls whilst C57 PhB-exposed dams exhibited less nursing behaviour (Laviola, *et al.*, 1990).

These studies have shown that certain drugs disrupt maternal behaviour and imply that disturbance to maternal care or the delicate mother-pup relationship may be the cause of differential patterns of behaviour in the offspring rather than direct effects of prenatal exposure to a toxicant.

Alterations to the quantity or quality of maternal behaviour following exposure to Al during pregnancy was suggested as a possible explanation for the maternal treatment effect on pup weight reported in chapter 3. Only one previous study has included simultaneous recording of maternal behaviour after prenatal exposure to Al (Bernuzzi, Desor and Lehr, 1986). These authors reported no significant differences between treatment groups in nest-building, retrieval of pups to the nest or time spent with the young

following exposure to $AlCl_3$ (160 or 200mg Al/kg body weight/day) in the dam's diet from day 8 to 20 of gestation.

The experiment presented in this chapter was undertaken to characterise any changes to the pattern of maternal behaviour of control and Al-treated mothers. A comprehensive array of behaviours was considered to enable assessment of the effects of Al exposure not only on the overall pattern of maternal behaviour but also on individual components of it.

Some of the work described in this chapter has been submitted for publication (Rankin, Laviola, Valanzano, Alleva and Manning, submitted, see Appendix E).

5.2 MATERIALS AND METHOD

5.2.1 Animals and treatment

All mice were maintained in an air-conditioned room at 21±1°C and 60±10% relative humidity, with a 12 hour light cycle. Pellet food (enriched standard diet purchased from Piccioni I-2500 Brescia, Italy) and tap water were freely available.

Pregnant females from the two inbred strains were randomly divided into control and treated groups. Treated females were exposed to $Al_2(SO_4)_3$ by intraperitoneal injection, at a dose of 200mg/kg body weight during gestation days 10 to 13 inclusive. Maternal weights and the amount of food and water ingested were determined during this period.

5.2.2 Maternal Behaviour

Following cross-fostering on Pd1, as described in chapter 2 (see section **2.2**), each mother was housed in a clear plastic cage to facilitate the observation of maternal behaviour. The cage contained wood shavings and 1g of nesting material consisting of strips of paper. The cages were not cleaned until maternal behaviour observations were completed. The procedure employed for maternal behaviour observation was based on that described by Laviola, *et al.*, (1990), with 10 minute recording sessions twice daily (during the initial and final hours of the dark phase) under red light and randomised so that the observation of different treatment groups were equally represented at all times of the day. Dams were placed

in the experimental room 30 minutes prior to commencement of recording and remained there until the end of the day. The cage lids were not replaced by perspex for the observation periods for two reasons. Firstly, pilot studies showed that the changeover disturbed the mother and increased her exploration of the new cage lid. Secondly, leaving the standard lid made it possible to include the recording of *eating and drinking* behaviour. Although these behaviours are clearly not part of the maternal behaviour repertoire, I considered it important to record them in relation to the assessment of any influence of Al on milk production. Recording was continued to include a later age, day 18 postpartum, to investigate whether exposure to Al during gestation prolonged the expression of any behaviours.

Observations were made on postnatal days 2, 3, 5, 7, 10, 14 and 18 but not on Pd1 due to the possible disturbance of cross-fostering. These time periods are thought to correspond with major developmental and neurochemical changes within the infant mouse (Wahlsten, 1974). Behaviour was recorded on a keyboard linked to a computer using the programme "Key behaviour" (Deag, 1983a). The information was stored on computer discs for subsequent analysis (Deag, 1983b). The following elements of maternal behaviour were recorded:

Pup-directed behaviours

Nursing - The mother adopts the classic lactation position, arching her body over the pups. Nursing was recorded even when the mother

was seen in the nest grooming herself but having at least one pup attached to a nipple. This was common during the later ages.

Licking - Scored when the mother is solely actively engaged in licking any part of a pup's body.

Nest-building - This is recorded when the mother is in the nest and pulling shavings into it, is carrying material to the nest or is using her forepaws to push material towards it.

Retrieving - The mother returns a pup which had wandered from the nest site. In a laboratory situation such as this, retrieval of a pup is rarely seen.

If the mother was *nursing* but also engaged in one of the other pup-directed activities, the former was scored. I felt it was important to record any actual nourishment received by the pups in preference to other maternal activities occurring at the same time, to estimate if the pups were being affected by postnatal events.

Non pup-directed activities

Active - The mother is out of the nest and is moving around the cage.

Rearing - The mother is on her hind legs leaning against the cage walls.

Grooming - The mother is licking any part of her own body.

Still - The mother is lying still with no pups attached either beside the nest or at the other end of the cage.

Eating and Drinking - The mother is eating from the food hopper or drinking from the water bottle.

At the end of the 10 minutes recording period the quality of the nest was scored on a scale of 0 to 4; a score of 0 was given when there was no discernible nest site and the pups were scattered, and a nest was judged to have a score of 4 when it was well constructed and the pups were not obviously visible.

5.3 RESULTS

5.3.1 Estimated level of aluminium exposure

Pregnant females injected i.p. with $Al_2(SO_4)_3$ received 0.41mg of elemental Al per day, a total of 1.64mg over the four days of treatment, as reported in chapter 3.

5.3.2 Maternal weight

In the CBA strain there were 14 control and 15 treated pregnant females and 9 control and 12 treated females in the C57 strain. The greater number of CBA females resulted from the experiment being set up twice due to cannabalism of fostered young. For both strains weight gain during the treatment period (Gd10 to 13) was slight but increased greatly after its termination. Control and

treated females gained weight at a comparable rate in both strains, although CBA control females weighed about 5% more than treated females by day 18 of gestation.

As with prenatal weights, the weights recorded for control and treated dams from day 3 postpartum to weaning were not significantly different either between control and treated groups or strains.

Following injection of Al some CBA females showed dragging of the hindlimb, although this paralysis was temporary and the limb recovered a few days after treatment ended. This was not observed in C57 females.

5.3.3 Food and water intake

There were no differences in the amount of food and water ingested by control and treated CBA females during gestation days 10-18. Food intake was similar for control and treated C57 females. However water consumption was slightly reduced for treated C57 females (control n=9 $\bar{x}$=77.39($\pm$2.80)g, treated n=12 $\bar{x}$=68.23($\pm$1.77)g, $t(14)= 2.76$ $p<0.02$).

5.3.4 Maternal Behaviour

For purposes of analysis, maternal behaviour scores for the two daily sessions were combined, although there was a slight tendency towards an increase in *nursing* behaviour during the morning session. Figs. 5.1 - 5.6 summarise the most important differences in maternal activities for control and treated mothers over

the 7 test days for CBA (7 control and 6 treated) and C57 (6 control and 8 treated) females. The graphs represent the mean duration and frequency of bouts. Although individual comparisons of each behaviour between treatment groups were analysed using nonparametric tests (Mann Whitney U-test), means are presented rather than the medians due to the frequent occurrence of zero scores. Pup retrieval was not observed on any test day.

As with the Laviola, *et al.*, (1990) study and others (Ewart and Cutler, 1979; Chapman and Cutler, 1983) maternal care was, in general, more pronounced during the first few days after birth in both groups and for both strains, and subsequently declined as the pups matured, being replaced by non pup-directed activities. Although there were few significant differences between control and treated mothers for any of the behaviours measured, the graphs reveal some clear and consistent trends which will be described below.

Nursing - The frequency of bouts of *nursing* was similar for control and treated CBA females during the test period. The duration of *nursing* behaviour decreased as a function of time in both treatment groups. However, control CBA dams spent an increased amount of time *nursing* their offspring compared to treated females; this difference was more pronounced during the later test days (see Fig. 5.1).

From days 3 to 10 postpartum Al-exposed C57 mothers showed an increased number of bouts of *nursing* which were of longer duration than control mothers reaching marginal significance on Pd5 (see Fig. 5.2).

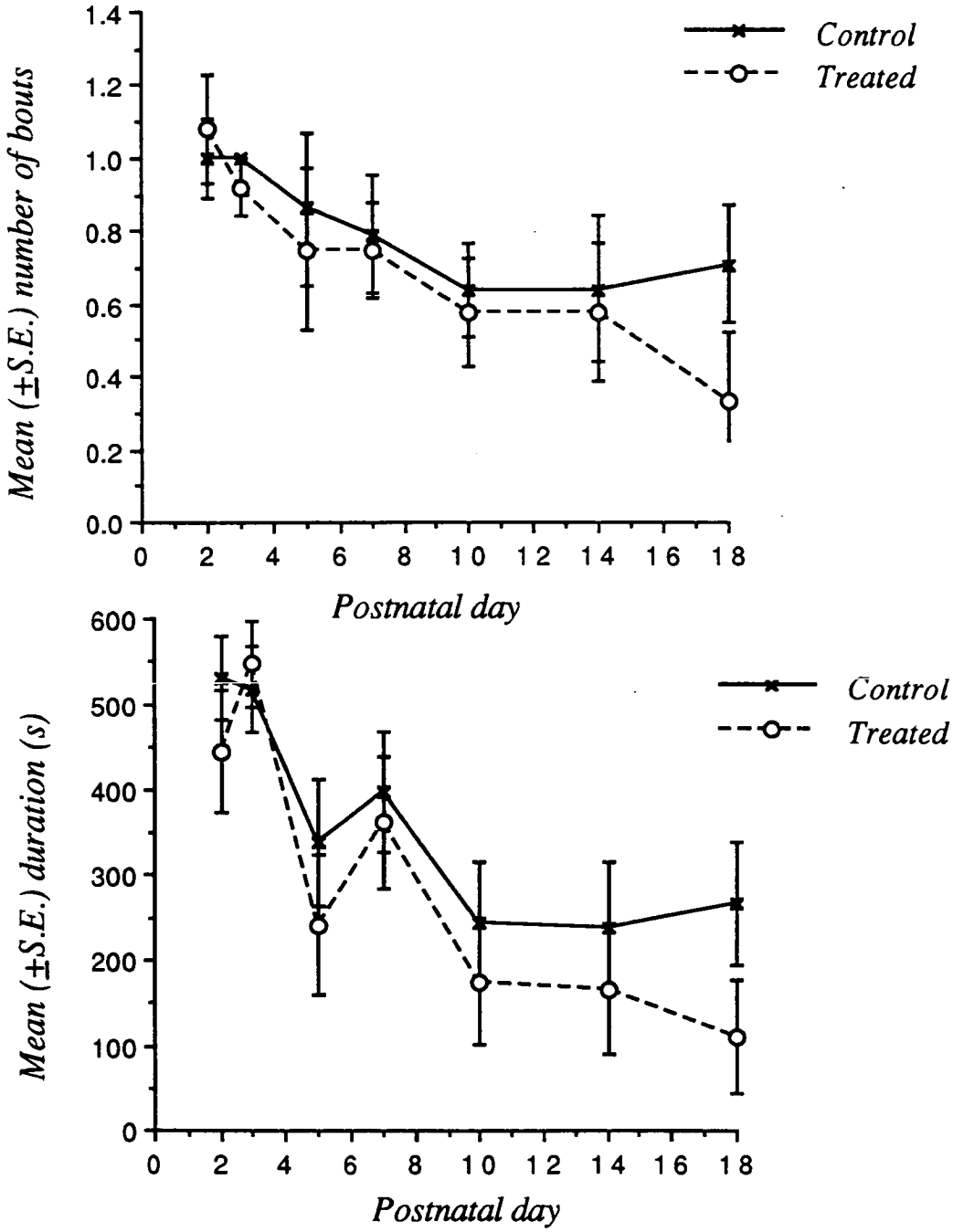


Fig. 5.1: Effect of gestational exposure to aluminium sulphate (200mg/kg, Gd10-13) on the nursing behaviour of CBA mothers.

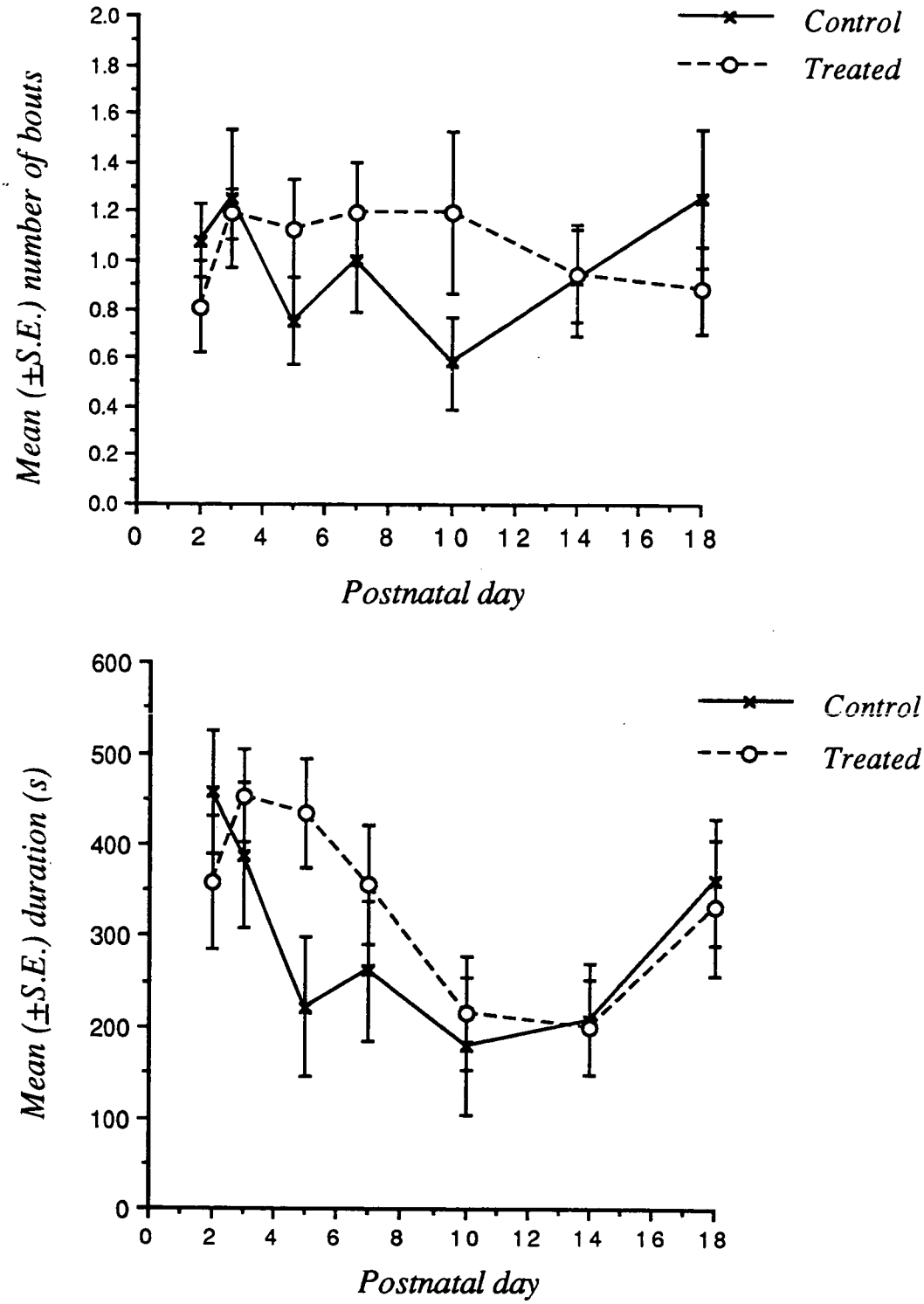


Fig. 5.2: Effect of gestational exposure to aluminium sulphate (200mg/kg, Gd10-13) on the nursing behaviour of C57BL/6J mothers.

Licking - Figs. 5.3 and 5.4 show that low levels of *licking* behaviour were recorded for both control and treated mothers in both strains throughout the test period. On Pd10 and 14 CBA treated females exhibited an increased number of bouts of *licking* of longer duration than control CBA females, which were significantly different on Pd14 (frequency $U=56.0$ $p<0.03$; duration $U=56.0$ $p<0.03$).

The frequency and duration of *licking* behaviour on all test days except Pd18 were slightly greater for treated C57 mothers than controls. For both control and treated mothers *licking* behaviour increased as a function of age and peaked on Pd10.

Nest-building - Very low levels of this behaviour were exhibited by either control or treated females from either strain.

As described earlier, non pup-directed behaviours were rare during the first postnatal week but became more predominant thereafter.

Active - The frequency and duration of bouts of *active* behaviour increased from Pd7 onwards for both control and Al-exposed CBA females. On Pd14 treated CBA females showed an increased number of these bouts which were of longer duration.

C57 mothers did not show such a clear pattern of *active* behaviour, although the frequency and duration of this behaviour was greater from Pd10 onwards. As with CBA Al-exposed females, treated C57 females showed an increase in the number and duration of bouts of *active* behaviour compared to controls.

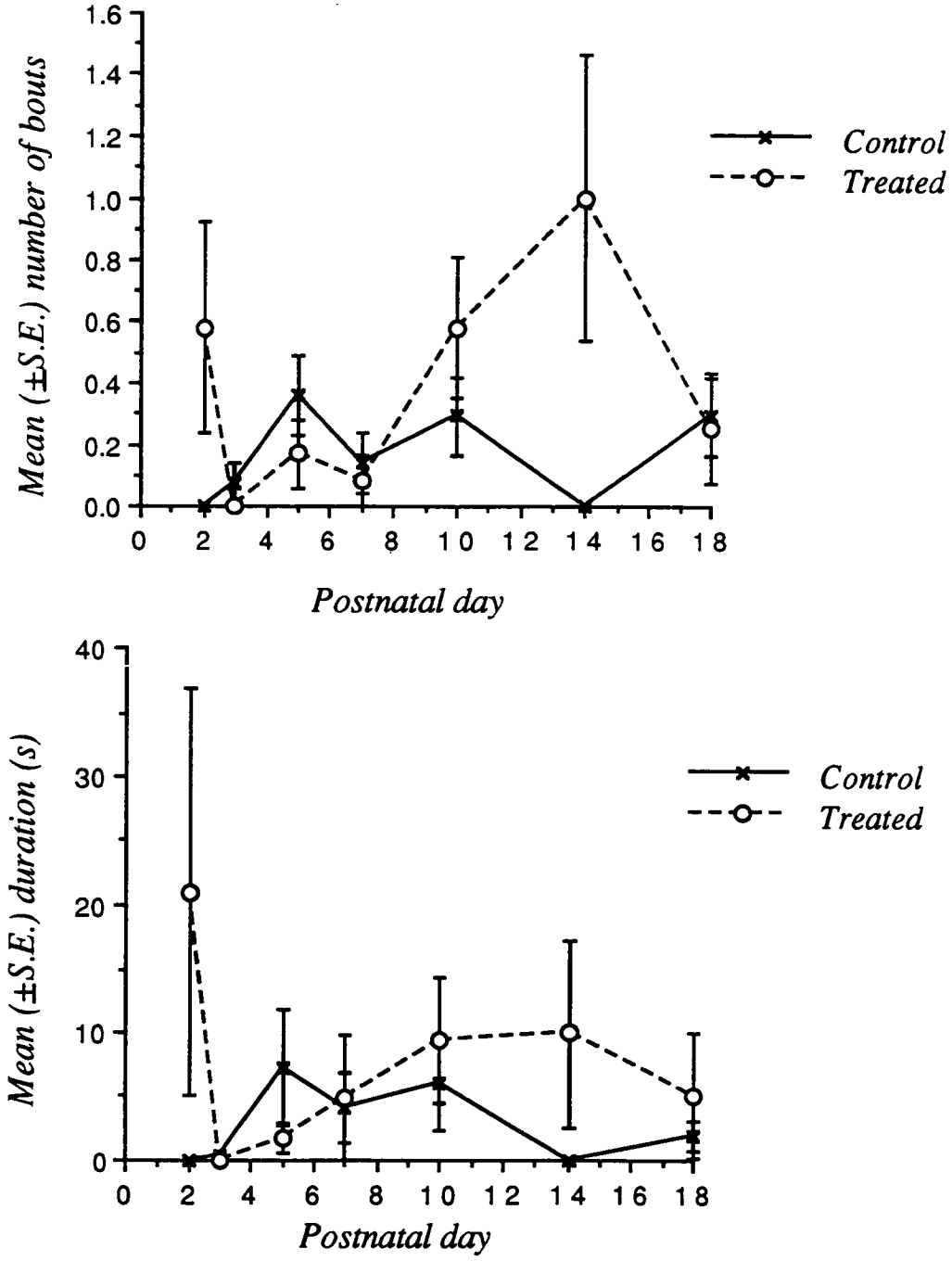


Fig. 5.3: Effect of gestational exposure to aluminium sulphate (200mg/kg, Gd10-13) on the licking behaviour of CBA mothers.

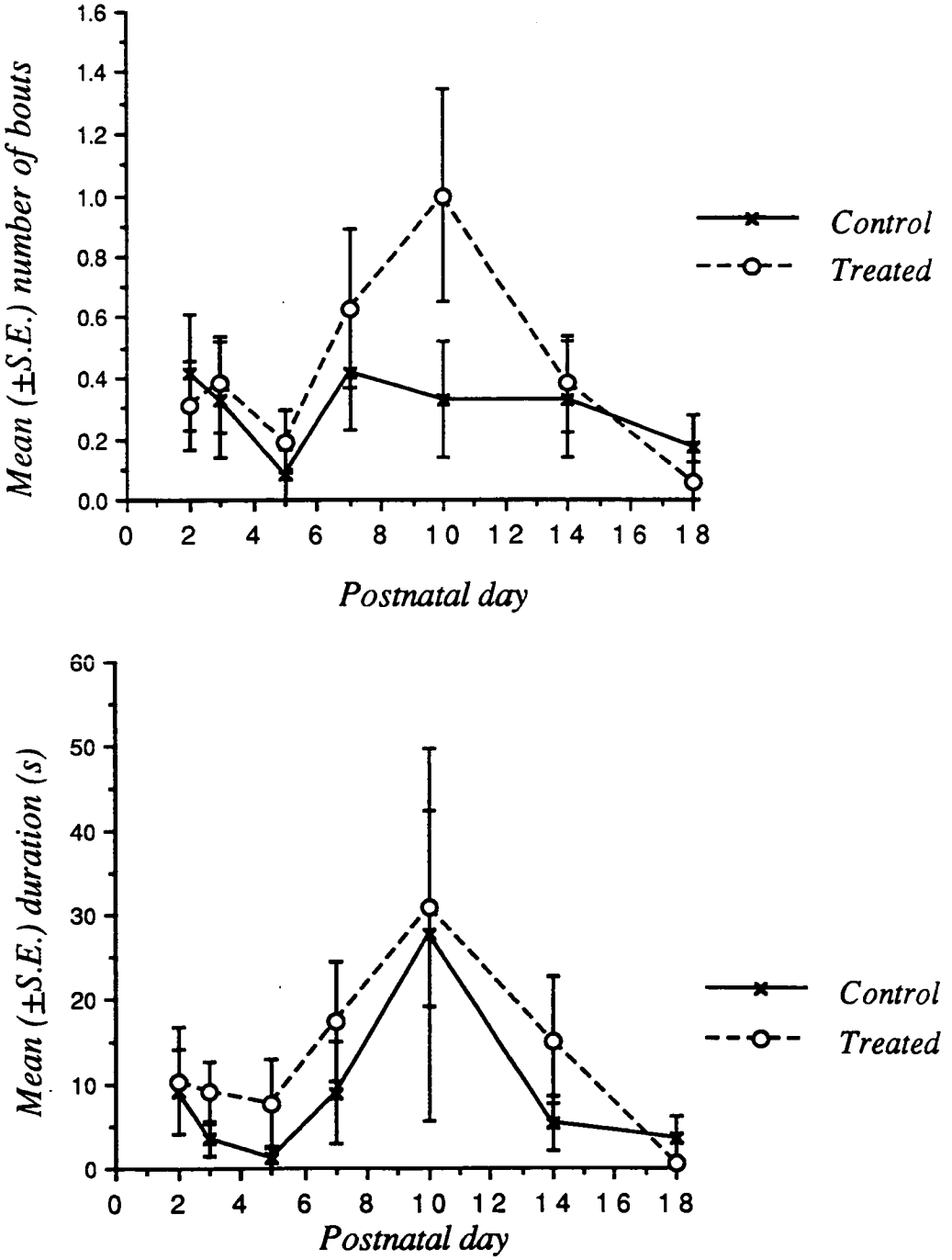


Fig. 5.4: Effect of gestational exposure to aluminium sulphate (200mg/kg, Gd10-13) on the licking behaviour of C57BL/6J mothers.

Still - Mothers of both strains and both groups all showed an increased frequency and duration of bouts of *still* behaviour during the second postnatal week. Control CBA females tended towards a greater amount of this behaviour during Pd7, 10 and 14.

The frequency of *still* behaviour was similar for C57 control and treated mothers, although control C57 females spent more time involved in this behaviour.

Groom - The frequency and duration of bouts of *grooming* behaviour increased as a function of age for CBA control mothers. However, Al-exposed CBA females showed a significant increase in the frequency ($U=42.0$ $p<0.03$) and duration ($U=39.5$ $p<0.02$) of *grooming* behaviour during Pd5.

The frequency and duration of *grooming* behaviour was similar throughout the test period for control and treated C57 females except on Pd5 when control C57 mothers spent more time involved in *grooming* ($U=133.0$ $p< 0.04$).

Rearing - The frequency and duration of *rearing* behaviour increased from Pd3 onwards for CBA mothers and both control and treated groups showed similar levels of this behaviour. However, on Pd14 Al-exposed CBA mothers showed a significantly greater number of bouts of *rearing* ($U=43.0$ $p<0.03$) and of longer duration ($U=41.0$ $p<0.02$) than controls.

The frequency of *rearing* for both C57 control and treated groups declined between Pd2 to 7 and then increased. Treated C57 females showed a greater number of bouts of *rearing* which were of longer duration than control mothers.

Eating and Drinking - Control and treated CBA mothers showed similar frequency and duration of bouts of *eating* and *drinking* which increased slightly from Pd3 to 18 (see Fig. 5.5). However, on Pd10 treated CBA females exhibited an increased frequency of bouts of *eating and drinking* which were of longer duration, but this latter difference was only marginally significant.

The frequency of food and water ingestion increased from Pd2 to 7 and then declined for C57 control females but steadily increased from Pd5 for Al-treated C57 mothers (see Fig. 5.6). Control C57 females ingested more food and water during Pd5-10 than treated females.

5.3.5 Nest quality score

The total scores of nest quality over the 7 test days showed that both control and treated CBA and C57 mothers built nests to a similar standard (CBA control $\bar{x}$=39.71($\pm$1.29), treated $\bar{x}$=40.5($\pm$1.43); C57 control $\bar{x}$=41.83($\pm$1.01), treated $\bar{x}$=40.25($\pm$1.06)).

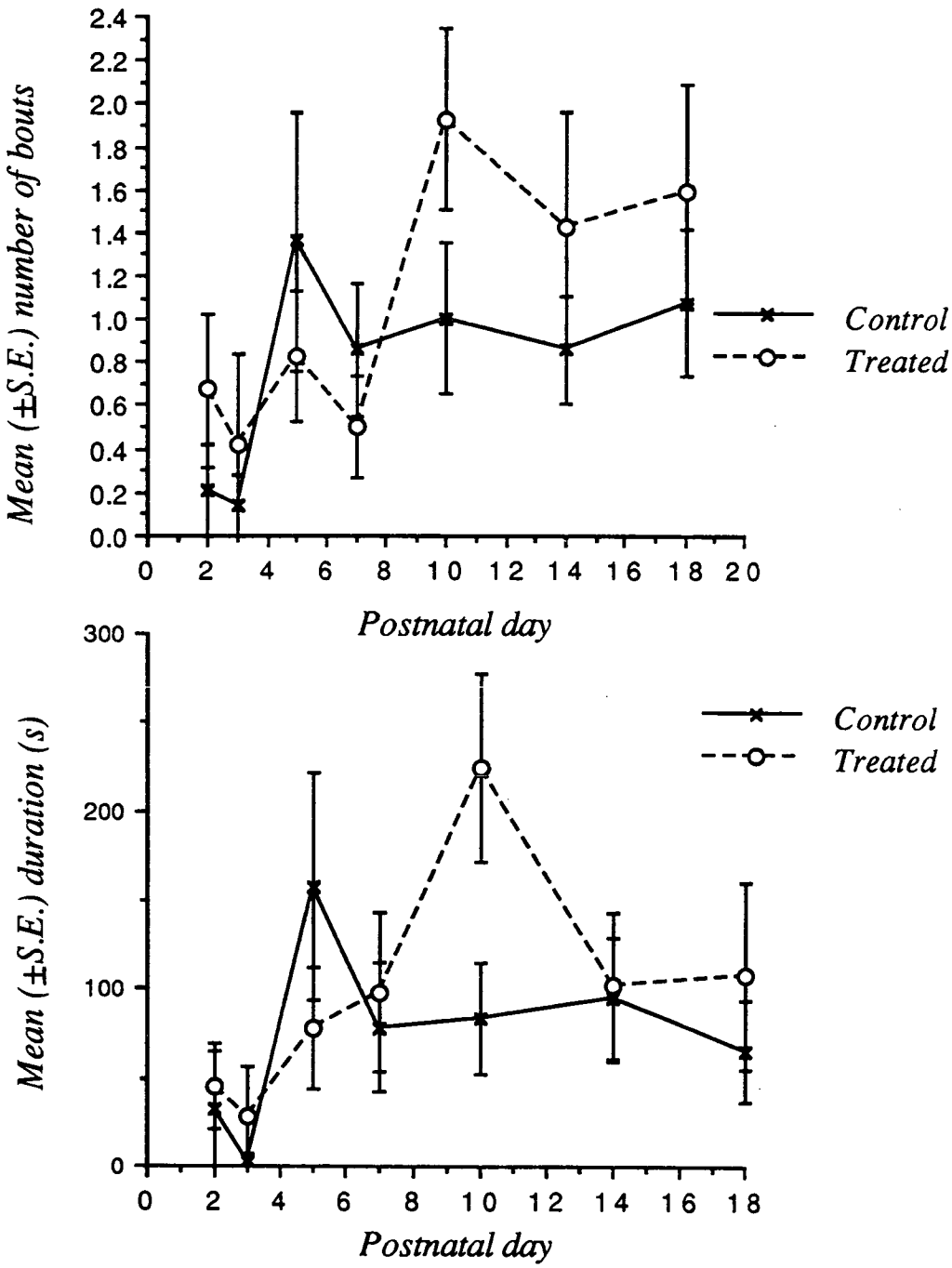


Fig. 5.5: Effect of gestational exposure to aluminium sulphate (200mg/kg, Gd10-13) on the eating and drinking behaviour of CBA mothers.

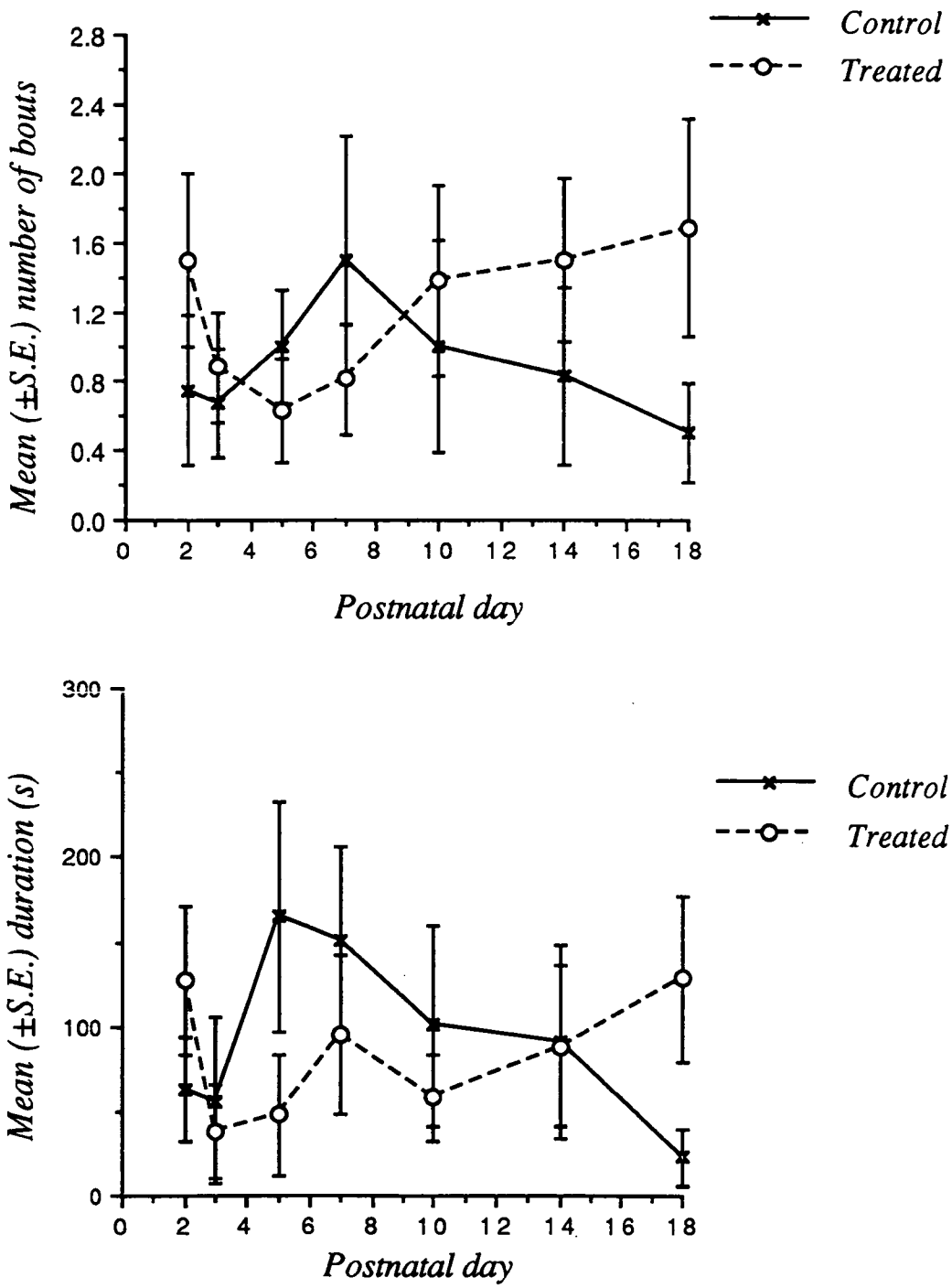


Fig. 5.6: Effect of gestational exposure to aluminium sulphate (200mg/kg, Gd10-13) on the eating and drinking behaviour of C57BL/6J mothers.

5.4 DISCUSSION

The results of this set of observations, which examined the effects of exposure to Al during a limited period of gestation on a number of maternal activities, revealed only slight differences between control and Al-treated mothers and then only on certain days. When a large number of comparisons are made on a varied set of data, some significant differences are bound to occur by chance.

The pattern of some components of maternal behaviour depended both on prior treatment and on the strain of the mother, whilst others were only treatment dependent. Al-exposed CBA mothers spent less time involved in *nursing* their offspring whilst C57 treated dams showed enhanced *nursing* behaviour compared to controls. However in the case of *licking*, the Al-treated mothers from both strains spent more time involved in this behaviour. The low level of *licking* behaviour may have resulted from the preferential recording of *nursing* behaviour if both behaviours occurred simultaneously. CBA treated mothers tended towards increased involvement in non pup-directed activities.

Hence, only slight differences in certain elements of maternal behaviour were found which are unlikely to reflect alterations in the ability of the mother to respond maternally towards her young. Exposure to Al did not affect the mechanisms responsible for the expression and organisation of maternal behaviour, which is in accordance with the results of Bernuzzi, *et al.*, (1986). Presumably the level of pup stimulation was equal for control and treated dams as each litter consisted of two control and two Al-treated pups. There

is no evidence to suggest a decrease in the ability of the pups to elicit adequate maternal responses.

The impairment in weight gain by CBA pups fostered to treated mothers described in chapters 3 and 4 can not be explained by deficits in availability of nourishment, as treated CBA mothers showed similar levels of *nursing*, *eating and drinking* behaviour to the controls. Indeed, the lower weight of C57 pups found in the experiment reported in chapter 4, can certainly not be explained in this way, as C57 treated mothers actually spent more time *nursing* and *eating and drinking*. However, although mothers were seen in the *nursing* position, lying with an arched back over the pups, the possiblity exists that the pups were not always actively engaged in suckling or that the milk is of the same quality.

There was only a slight difference in the amount of food and water ingested by control and treated CBA and C57 mothers. Thus, the body weight differences did not result from undernourishment of the treated mothers which has been shown to result in less efficient retrieval of young and in a reduction in *licking* (Smart and Preece, 1973).

Some studies involving prenatal exposure to alcohol, have found the greatest disturbance to certain components of maternal behaviour on Pd1 (Abel, 1978; Ewart and Cutler, 1979). In this study observations were not made on Pd1 as it was considered that the cross-fostering procedure may have disturbed the mother and altered the natural pattern of maternal behaviour on this day. Perhaps further group differences would have been found if this day had been included.

Despite the lack of significant effects of exposure to Al during gestation on maternal behaviour, Al had toxic effects on other maternal characteristics. In both this experiment and the one described in chapter 3, pregnant CBA females exposed to Al via i.p. injection gained less weight during gestation than did the control females, although this difference was not significant in this experiment. In addition, a temporary hindlimb paralysis was observed in some CBA females following i.p. injection with Al. This has been previously reported in Swiss-Webster mice following exposure to 1000ppm aluminium lactate in the diet from day 0 of gestation to weaning, although it was not evident until days 12-15 postpartum (Golub, Gerschwin, Donald, Negri and Keen, 1987).

The degree of maternal care was not detectably different between control and treated mothers using these measures. This leads to the conclusion that the impairments in body weight and neurobehavioural development of pups reared by treated mothers are unlikely to be explained in terms of an alteration to the mother's behaviour alone. Due to the impairment in maternal weight gain during gestation for Al-exposed females, one can not exclude the possibility that these differences resulted from this initial impairment in maternal weight. Ness and Franchina (1990) have suggested that any dimunition in female body weight and nutritional intake may be of more consequence to the offspring if it occurs during lactation. Control and Al-treated maternal weights did not differ during the postnatal period, so perhaps the influence of any decrease in body weight which occurred prenatally was overcome.

The unlikely contribution of differences in maternal care points to a direct effect of Al on the physiology of the mother.

Exposure to Al during pregnancy has been shown to reduce milk consumption of rabbit offspring in a dose-dependent manner (Yokel, 1984; Yokel, 1985). This is the case for both treated and control pups suckling from Al-exposed lactating does. Thus, the maternal treatment effect is not likely to be mediated via changes in the ability of the Al-exposed mothers to care for their young but effects of Al on milk production may be of greater relevance. Moreover, the nutritional value of the milk produced by Al-exposed mothers may be inferior to that produced by control mothers.

Another possible explanation for the decreased body weight of pups reared by treated mothers is that Al exerts its effects directly on the pup. A decrease in milk yield may be accompanied by a decline in suckling attempts by offspring fostered to treated mothers. As milk output and Al content were not assessed during this experiment these possibilities remain speculative.

5.5 EXPERIMENT 2

Effect of a scopolamine challenge on CBA and C57BL/6J pup locomotor activity at weaning.

5.5.1 Introduction

Experiment 2 of chapter 3 (section **3.6**) described the use of a challenge with scopolamine to bring out any differences in maze learning between control and Al-treated CBA male mice. In this experiment it is used again but this time to investigate differences in the locomotor activity of control and treated mice.

The use of pharmacological challenges to uncover behavioural differences resulting from prenatal drug treatments which are too subtle to be detected using conventional behavioural tests, has become common practice in behavioural teratology (Walsh and Tilson, 1987; Hannigan and Blanchard, 1988; Spear, 1990). As discussed in chapter 3, behavioural differences may not become manifest due to the inherent plasticity of the CNS, referred to as "*the functional reserve*" by Walsh and Tilson (1987), which may obscure differential responses unless the system is further stressed. For example, the activity levels of CD-1 mice, whose mothers were exposed to 0.5% lead acetate in their drinking water during lactation, was similar to that of controls when tested at 211 days of age. However, the hyperactive response following a challenge with apomorphine (4.0mg/kg) was attenuated compared to that of controls (Rafales, Bornschein, Michaelson, Loch and Barker, 1979).

Both the cholinergic and dopaminergic neurotransmitter systems are important in the control of movement (McGreer, Boulding, Gibson and Foulkes, 1961). A reciprocal balance exists between these two systems which if disturbed results in recognised clinical disorders e.g. Parkinson's disease and Huntington's chorea (Levin, McGurk, Rose and Butcher, 1990). The most commonly used pharmacological agents in drug challenges are amphetamine, apomorphine and scopolamine, all of which induce hyperactivity following exposure. The first two agents cause this enhancement of activity by stimulating dopamine release and the latter by reducing the inhibitory control of the cholinergic system on activity (Laviola, Renna, Bignami and Cuomo, 1988). Scopolamine is the agent of choice in the experiment reported herein.

As the pharmacological action of the agent on specific neurotransmitter systems is known, drug challenges not only play a role in unmasking behavioural differences, but also help to further understand the neurochemical basis of such differences.

The aim of the experiment described here was to further investigate the effects of Al on locomotor activity in the two inbred strains of mice by challenging these mice with scopolamine. As previous studies have shown that the characteristic hyperactivity induced by scopolamine is not evident until the end of the third postnatal week (Campbell, Lytle and Fibiger, 1969; Alleva and Bignami, 1985), pups were challenged with scopolamine at weaning (Pd21).

5.6 METHOD

5.6.1 Treatment

The subjects for this experiment were the offspring of the dams used as experimental subjects in the maternal behaviour observations reported in chapter 5. At weaning (Pd21) subjects were randomly divided into two groups one of which received a single intraperitoneal injection of scopolamine hydrochloride (0.5mg/kg body weight), and the other received the equivalent volume of saline. The dose of scopolamine was chosen on the basis of previous studies (Alleva and Bignami, 1985; Alleva, Aloe and Laviola, 1986).

5.6.2 Procedure

Pup activity was recorded for three minutes on Pd15 and 18 as described in chapter 2 (*2.2.1*). On Pd21 mothers were removed from the cages and each subject injected as designated. Following injection the animal was placed in a cage in the experimental room for 15 minutes and then into a clean cage in a Varimex activity meter apparatus (4 units Columbus Instruments) set at a standard level. The recording session lasted for 15 minutes, with activity being recorded automatically and printed out in 3 minute blocks.

5.7 RESULTS

The main ANOVA tables are given in Appendix F.

5.7.1 Pup activity

In all groups activity significantly increased between Pd15 and 18 as shown by the significant effect of day. The activity of 27 CBA pups (15 males and 13 females) was recorded. In contrast to the results presented in chapter 3 (see section *3.4.8*), the effect of prenatal treatment with injected Al on CBA activity was not sex dependent. There was a slight trend towards treated pups being less active than controls but this was not significant (see Fig. 5.7). There was a significant interaction between day, prenatal treatment and foster mother treatment on CBA activity ($F(1,19)=6.47$ $p<0.02$).

There was a total of 55 pups of the C57 strain, 30 males and 25 females. The activity of these pups is shown in Figs. 5.8a and b respectively. There was a significant interactive effect of prenatal treatment and sex on C57 pup activity ($F(1,47)=5.63$ $p<0.03$) and of these variables and postnatal day ($F(1,47)=6.69$ $p<0.02$). Separating the data by sex revealed that males exposed to Al *in utero* were less active than controls, although this difference only reached marginal significance. There was a significant interactive effect of day and prenatal treatment on male activity ($F(1,26)=4.22$ $p=0.05$), this difference in activity was more pronounced on Pd18. In the case of C57 females, there were no differences in activity levels on Pd15 but on Pd18 the trend was in the opposite direction from that of males; prenatally Al-treated females were more active than controls, although this difference did not reach significance.

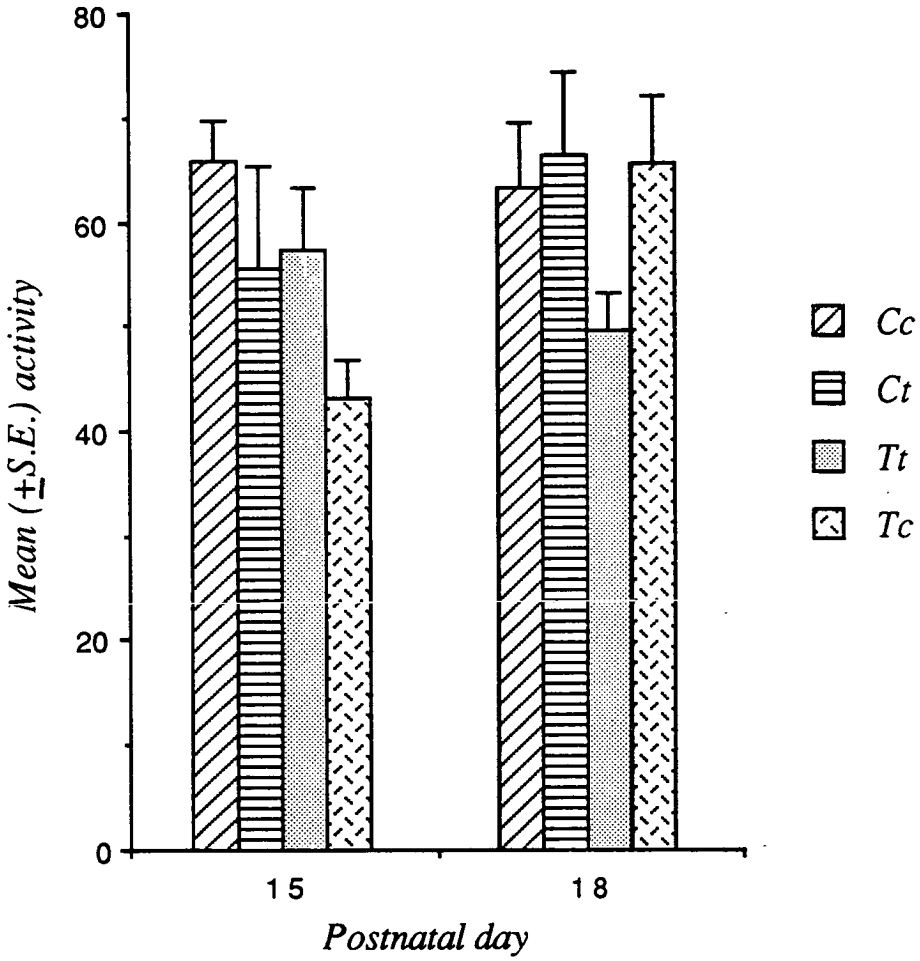


Fig. 5.7: Effect of prenatal exposure to aluminium sulphate (200mg/kg, Gd10-13), on the number of squares crossed by CBA pups in a three minute openfield test.

Fig. 5.8a

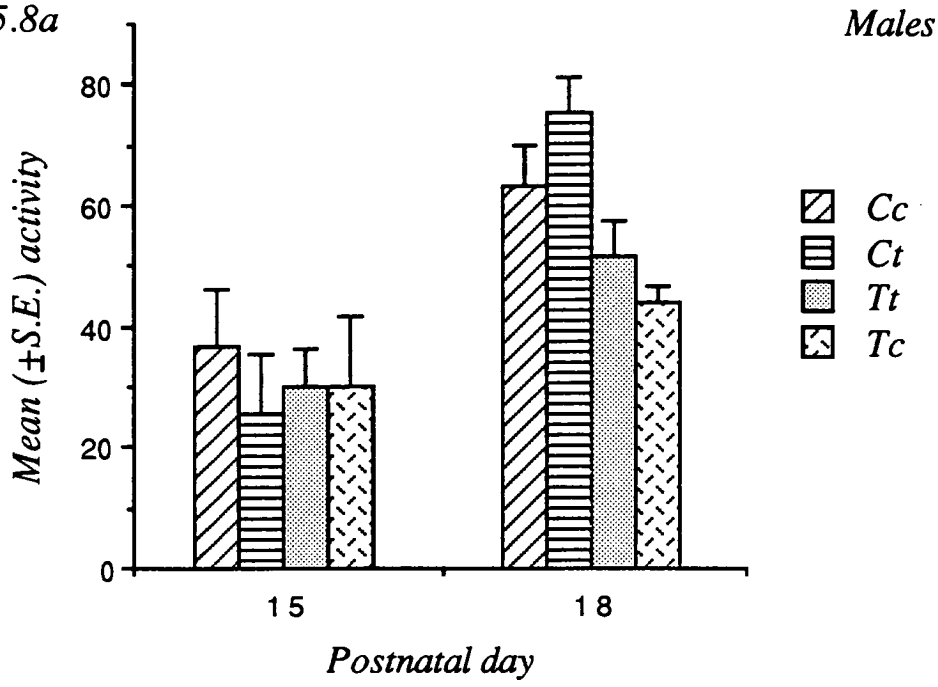


Fig. 5.8b

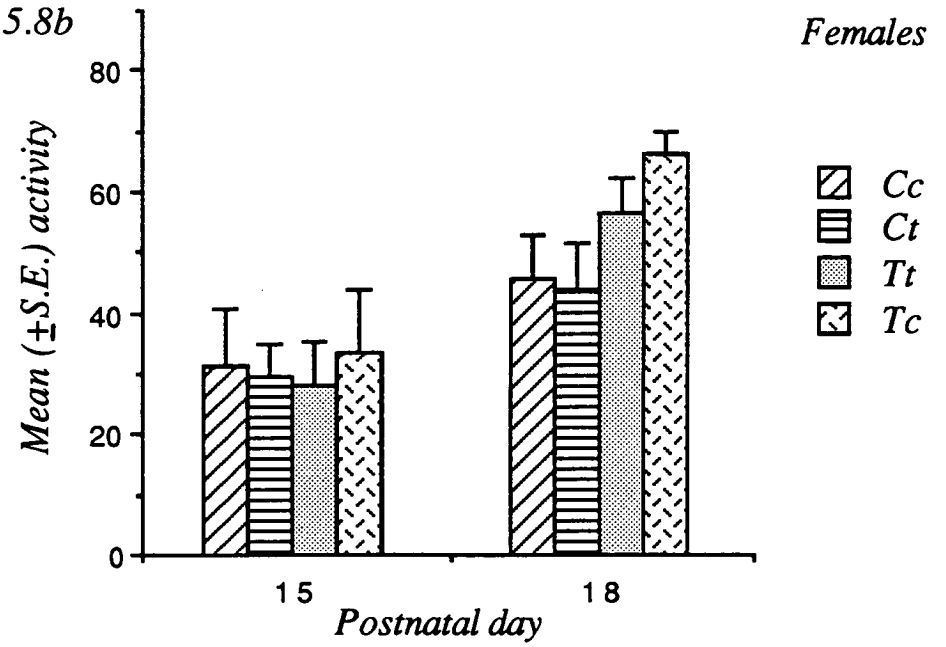


Fig. 5.8: Effect of prenatal exposure to aluminium sulphate (200mg/kg, Gd10-13) on the number of squares crossed by C57 BL/6J pups in a three minute openfield test.

5.7.2 The effect of scopolamine on activity at weaning

All subjects showed within-session habituation, irrespective of strain or treatment, as evidenced by the decrease in activity levels as the test proceeded. Activity was recorded in 3 minute blocks and this was the repeated measure.

As noted in section *5.3.2*, the experiment was set up twice in the case of the CBA strain. The first group of CBA animals were not tested at Pd15 and 18 but all were exposed to scopolamine. There were a total of 38 CBA animals. A challenge with scopolamine produced the expected enhancement of activity in each of the CBA experimental groups ($F(1,30)=64.91$ $p<0.0001$, see Fig. 5.9). There was a significant interaction between prenatal treatment and the drug challenge ($F(1,30)=5.19$ $p=0.03$), the potentiation of activity being less in those pups which had been exposed to Al *in utero*. There was a significant interactive effect of scopolamine and the repeated measure (total activity in 3 minutes) ($F(4,120)=3.3$ $p<0.02$), the effects of scopolamine declining with time. The significant effect of the repeated measure ($F(4,120)=23.33$ $p<0.0001$) shows a within-session habituation, as activity decreased with time.

Within the C57 strain, male and female pups were analysed separately due to the significant interaction between prenatal treatment and sex ($F(1,39)=8.94$ $p<0.005$) and between these and the repeated measure ($F(4,156)=2.82$ $p=0.04$).

Figs. 5.10a and b summarise the effects of scopolamine on C57 male and female activity respectively. There was a significant effect of prenatal treatment on C57 male activity ($F(1,22)=4.82$

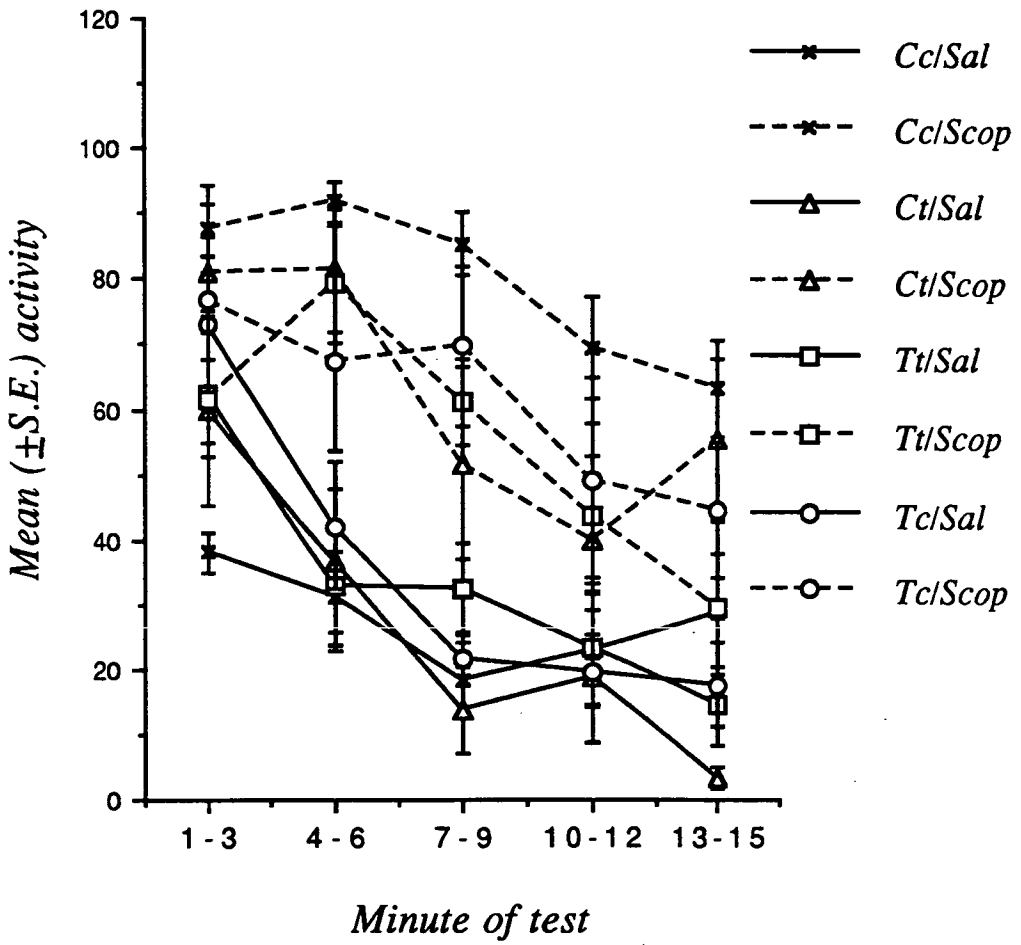


Fig. 5.9: Effect of a scopolamine challenge on CBA pup activity at weaning.

$p<0.04$), treated males exhibited diminished activity levels compared to controls, and a significant interactive effect of the repeated measure and prenatal treatment ($F(4,88)=2.93$ $p<0.04$). The scopolamine challenge had a significant effect on male activity ($F(1,22)=11.72$ $p<0.003$), but interestingly the direction of influence was opposite to that found in the CBA strain; scopolamine failed to produce the expected enhancement in activity but rather reduced activity levels in all experimental groups. This effect of scopolamine diminished with time, as shown by the significant interaction of the challenge and the repeated measure ($F(4,88)=6.02$ $p<0.002$). Foster mother treatment, the challenge and the repeated measure had a significant interactive effect on C57 male activity ($F(4,88)=3.58$ $p<0.02$); the reduction in activity was less pronounced for those pups fostered to control mothers.

The influence of prenatal exposure to Al on C57 females at Pd21 tended towards an increase in activity levels, although this difference was only marginally significant. As with C57 males and the CBA animals, C57 females showed within-session habituation ($F(4,68)=14.19$ $p<0.0001$). There was a significant effect of scopolamine on C57 female activity ($F(1,17)=6.15$ $p<0.03$), again scopolamine caused a reduction in activity compared to saline-exposed counterparts (see Fig. 5.10b). Although there was no significant interaction between prenatal treatment and the challenge, the scopolamine-induced hypoactivity was more pronounced for control females.

Fig. 5.10a

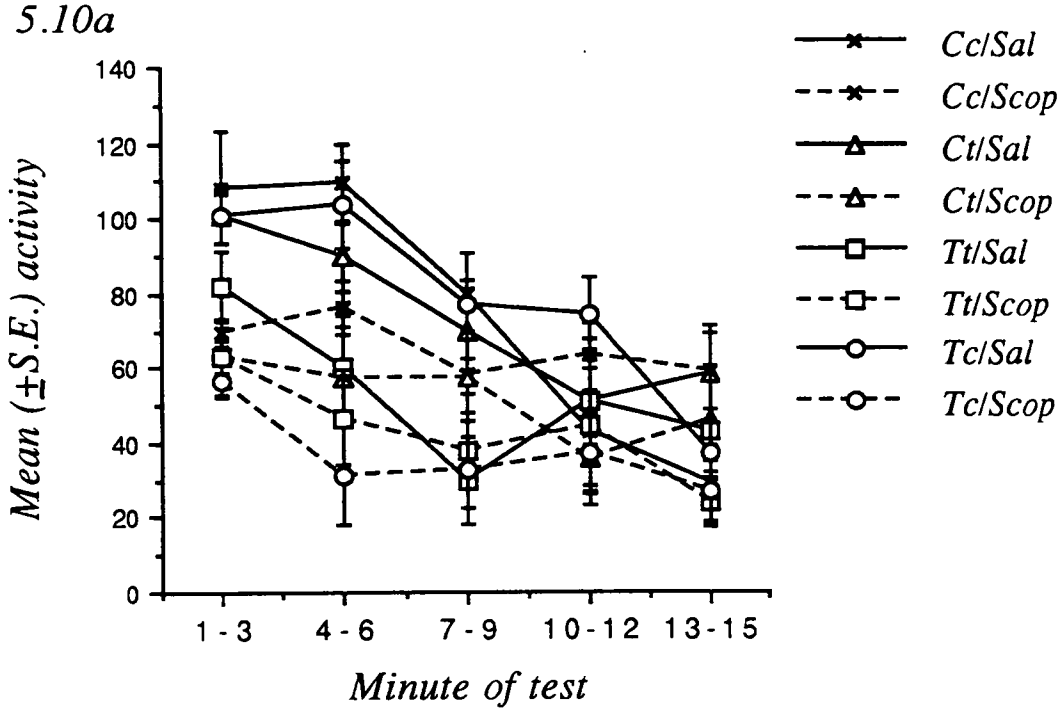


Fig. 5.10b

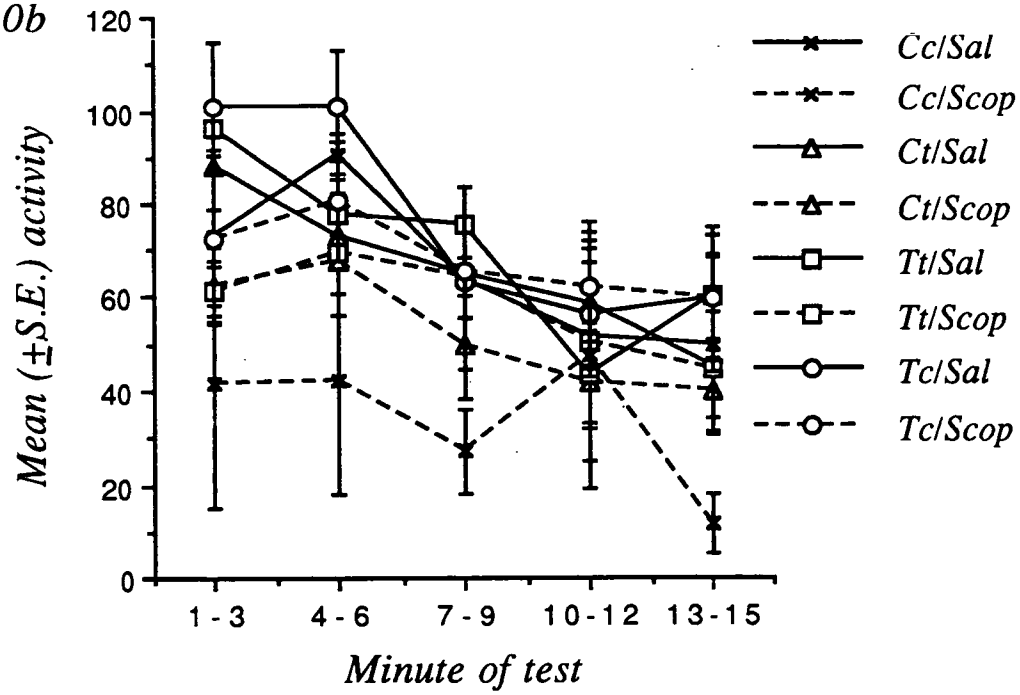


Fig. 5.10: Effect of a scopolamine challenge on C57BL/6J pup activity at weaning.

5.8 DISCUSSION

The results reported in chapter 3 suggested a differential effect of prenatal exposure to injected Al on CBA pup activity depending on sex; Al-exposed CBA females were less active than controls, whilst treated males exhibited enhanced activity levels. However, this finding was not repeated in the experiment reported here. In fact, there was a trend towards Al-treated CBA pups being less active than controls. This may have resulted from differences in sample size as there were fewer females in this experiment.

Although scopolamine produced the expected hyperactivity in control and prenatally Al-treated CBA animals, the enhancement was less for treated subjects, as revealed by the significant interaction between prenatal treatment and scopolamine. Perhaps in the case of treated subjects, prenatal exposure to Al resulted in a loss of muscarinic cholinergic (mCHOL) receptors so there were fewer present to be occupied by scopolamine. The levels of ChAT activity within the CBA strain were significantly reduced in the hippocampus and striatum at 3 weeks of age (see Table 3.1), which suggests that deficits within the cholinergic system had indeed occurred.

Sex differences in activity levels were found within the C57 strain; male pups were more active than females. C57 males exposed to Al *in utero* were hypoactive compared to controls. Conversely, treated C57 females were more active than controls. This hypo- and hyperactivity for C57 males and females respectively, was also present at Pd21.

In contrast to the effect on subjects within the CBA strain, exposing C57 animals to scopolamine resulted in a decrease in

activity levels. This hypoactivity was more pronounced for controls. Further, there was a maternal treatment effect on the diminution of activity; male pups fostered to control mothers showed a lower detriment in activity levels following exposure to scopolamine.

Clearly, the response to scopolamine was highly dependent on the genetic strain. Similar strain differences in susceptibility to scopolamine have been noted previously. van Abeelen and Strijbosch (1969) found that C57BL/6J animals exhibited a decrease in exploration following exposure to scopolamine at a dose of 1.25mg/kg, whilst mice from the DBA/2J and SEC/1ReJ inbred strains responded in the opposite direction to the same exposure.

As noted in chapter 1 (**1.2***v*), pharmacogenetic factors influence the response of different inbred strains to drugs. As well as differences in behaviour between inbred strains of mice, there are also differences in brain chemistry. For example, Pryor, Schlesinger and Calhoum (1966) measured brain acetylcholine esterase activity (AChE) in five inbred strains of mice. These authors found higher AChE activity within the brains of animals from the DBA/2 strain compared to C57BL/10 animals. Similarly, Tunnicliff, Wimer and Wimer (1973) reported an increase in whole brain ChAT levels in mice from the BALB/c and DBA/2 inbred strains and lower levels in C57BL/6J mice. Thus, the strain differences in response to scopolamine present in this experiment may be explained in terms of strain differences in brain neurotransmitters. For example, Kempf, Greilsamer, Mack and Mandel (1974) found that the levels of noradrenaline were higher in the whole brain of C57BL/6J mice compared to DBA/2J and SEC/1ReJ animals.

Challenging animals with scopolamine provides information on the functional status of the mCHOL system only. As only slight differences were found between prenatally Al-treated animals and controls, one must conclude that Al did not appreciably alter the maturation of the mCHOL receptors. On the other hand, there is no information concerning the functional maturation of the nicotinic cholinergic (nCHOL) system. Thus, it is conceivable that nicotinic receptors may have been compensating for any alterations due to Al treatment. Combined treatment with a muscarinic and nicotinic receptor antagonist may provide more detailed information concerning the maturation of the cholinergic system at this age following exposure to Al.

CHAPTER 6

Effect of gestational exposure to oral aluminium sulphate on CBA and C57BL/6J pup development.

6.1 INTRODUCTION

The most likely route of entry of Al into the human body is by oral exposure, which can come from several sources. Al is added as a flocculant in the treatment of water, the majority of pharmaceutical preparations containing Al are taken in the form of tablets and any addition of Al to food following cooking in Al utensils (Trapp and Cannon, 1981) will be ingested orally. In recent years several accidents have occurred in the U.K. (Cross, 1990) which have resulted in the levels of Al in domestic water supplies rising to between 30 and 620mg/L (Eastwood, Levin, Pazianas, Taylor, Denton and Freemont, 1990), far beyond that recommended by the EEC (approximately 0.2mg/L). As little is known about the bioavailability of orally ingested Al, the consequences of such exposure will only be fully recognised through future epidemological studies when any long-term problems have become manifest within the human population. One study of a human infant population reported an increased incidence of talipes among Al-exposed infants, but no differences in other measures of pregnancy outcome (Golding, Rowland, Greenwood, and Lunt, 1991). This paper did not report on any behavioural measures and further studies will be needed to assess any latent effects.

The effects of oral exposure to Al are of particular concern considering the mounting clinical evidence of Al loading in infants who are not receiving dialysis but were given antacids containing Al

(Griswold, Reznik, Mendoza, Trauner and Alfrey, 1983; Randall, 1983; Andreoli, Bergstein and Sherrard, 1984; Chedid, Fudge, Teubner, James and Simmer, 1991). In addition, Sedman, Klein, Merritt, Miller, Weber, Gill, Anand and Alfrey (1985) have shown that intravenous feeding of premature infants resulted in high plasma and urinary Al concentrations and a 10-fold increase of Al in bone. Further, the urinary Al concentration did not reach the control level until several weeks after parenteral feeding was stopped, suggesting that there had been an accumulation of Al in the infant's body. However, this finding in premature infants was not confirmed by Puntis, Hall and Booth (1986) who examined the plasma Al concentration in older infants (5-22 weeks of age) receiving parenteral nutrition. This suggests that it is only immature infants and those with inefficient renal functioning who are at risk from Al toxicity via this route. Infant milk formula has been implicated as another possible source of Al to which infants may be exposed (Freundlich, Zilleruelo, Abitbol and Strauss 1985; Bishop, McGraw and Ward, 1989): the Al content of infant formula per litre has been estimated to be 165 times that of breast milk (Weintraub, Hams, Meerkin and Rosenberg, 1986). Hence, young children may be susceptible to Al intoxication from these various sources which are in common clinical use. As Marquis (1982) recommends, the consequences of exposure of the developing foetus to oral Al warrants urgent investigation.

6.1.2 Oral exposure to aluminium compounds

The introduction to chapter 3 reviewed the literature on exposure to Al via intraperitoneal injection, this chapter considers effects on the developing animal of exposure to oral Al. When aluminium nitrate ($Al(NO_3)_3$) was administered by gavage to pregnant Sprague-Dawley rats during days 6-14 of pregnancy, a decrease in maternal weight gain and in foetal body weight resulted. In addition, the incidence of skeletal malformations was higher among treated foetuses (Paternain, Domingo, Llobet and Corbella, 1988). Similar results were reported when oral doses of $Al(NO_3)_3$, at levels not toxic to the mother, were administered to Sprague-Dawley rats during pregnancy and lactation, resulting in a decreased number of litters and surviving offspring and increased pup mortality. Body weight and length were reduced in the treated groups from birth to weaning (Domingo, Paternain and Llobet, 1987a; Domingo, Paternain, Llobet and Corbella, 1987b). Exposure of pregnant outbred Wistar rats to $AlCl_3$ in their diet from day 8 of gestation to birth (Bernuzzi, Desor and Lehr, 1986) or throughout gestation (Bernuzzi, Desor and Lehr, 1989), resulted in a decrease in maternal weight during gestation, increased pup mortality, reduced pup body weight and caused a transient delay in the maturation of negative geotaxis and the righting reflex. On the other hand, Muller, Bernuzzi, Desor, Hutin, Burnel and Lehr (1990) did not find any differences in maternal weight gain, postnatal mortality or pup body weight following exposure to aluminium lactate (AlLact) at a dose of 400mg/kg/day. However, Al-treated pups showed delays in the

righting reflex test, in locomotor coordination and in an operant conditioning test.

Toxic effects from gestational exposure to oral Al have also been found in the mouse. Exposure of pregnant mice of the Dobra Voda strain (a strain bred in this Polish laboratory) to $AlCl_3$ in their drinking water (19.3mg Al/kg/day) resulted in a dose-dependent retardation in growth of the offspring but no difference in the number of litters (Ondrieka, Ginter and Kortus, 1966). These offspring were exposed to Al at 4 weeks of age in a similar manner to their parents. The growth retardation was more pronounced in the second and third generation mice (Ondrieka, Ginter and Kortus, 1966). Oral administration of AlLact in the diet of pregnant Swiss-Webster mice (S-W) produced maternal toxicity and reductions in growth and developmental delays of treated offspring (Golub, Gerschwin, Donald, Negri and Keen, 1987). In a follow up study, S-W mice were exposed to AlLact in a supplemented diet, which did not cause a reduction in maternal weight gain during gestation and lactation. Treated offspring showed developmental delays in a vertical screen test, had greater foot splay distances at weaning, indicating muscle weakness, and a reduced thermal sensitivity (Donald, Golub, Gerschwin and Keen, 1989).

The following chapter presents the results of a series of experiments exposing pregnant mice to $Al_2(SO_4)_3$ in their drinking water. Zbinden (1981) has argued that in any teratological investigation of a substance, several doses must be considered before an adequate picture of an agent's potential teratogenicity can be drawn. It was hoped that this route of administration would not cause maternal weight loss and, in the case of the C57 strain, would

increase the number of mothers who continued to term so that a clearer estimate of Al's effects could be made.

Administering an agent via the oral route is bound to increase the variability between the doses taken in by each subject. For this reason the water bottles were weighed daily to calculate the amount consumed. In this chapter the results of exposure to oral Al at three different doses (750, 1000 and 1250mg/L) during gestation are presented. Not all the same behavioural and neurochemical measures were taken for each dose due to practical and time limitations, so details are given in each section.

6.2 METHOD

6.2.1 Treatment

Al was dissolved in water taken from the Mouse House at a dose of either 750, 1000 or 1250mg/L. The pH of the resulting solution was measured using a pH meter and control water was acidified to the same pH using a few drops of sulphuric acid (pH=4.1). Plugged females were randomly divided into two groups receiving either the Al solution or the acidified control water in their water bottles during gestation days 10-16 inclusive for the 750mg/L group, and from days 1 to 20 of gestation for animals exposed to the 1000 and 1250mg/L dose. Fresh Al solutions were prepared every other day to prevent Al aggregation, as has been reported to have occurred in other studies (e.g. Gulya, Rakonczay and Kasa, 1990). The water bottles were weighed daily in order to calculate water intake. Pre- and postnatal maternal weights were monitored.

6.2.2 Maternal behaviour

As the maternal behaviour observations conducted in chapter 5 revealed few differences between the pattern of maternal behaviour during the morning and afternoon sessions, the system was simplified here with only one set of observations in the morning but extended to a duration of 15 minutes. Nest quality score was not recorded.

6.2.3 Scopolamine challenge

The procedure for the scopolamine challenge described in section *3.7.2* was repeated here. Briefly, after reaching the criterion of two consecutive errorless trials in the maze, subjects were injected i.p. with scopolamine hydrochloride (0.5mg/kg body weight) 20 minutes prior to being retested.

6.2.4 Assay for choline acetyltransferase (ChAT) activity.

The results of experiments conducted in chapter 3 revealed an influence of foster mother treatment on body weight and several other developmental landmarks. For this reason, animals treated with oral Al at the 1000mg/L dose were divided into the four experimental groups and separated by sex, for analysis of ChAT activity at 23 weeks of age.

6.3 RESULTS

The following section incorporates the results of three separate experiments during which pregnant females from the two genotypes were exposed to oral Al at three doses (750, 1000 and 1250mg/L). The results are difficult to comprehend due to the number of strain and treatment comparisons as well as the diverse range of measures. With such a large number of comparisons involved, it is inevitable that significant differences may arise on, for example, one day only. This section aims at presenting the most important effects of exposure to oral Al, whilst the discussion will concentrate on general trends. The main ANOVA tables and median + lower and upper interquartile ranges are given in Appendix G.

6.3.1 Water and food intake

A summary of water intake is presented in table 6.1. Adding $Al_2(SO_4)_3$ at a dose of 750 and 1000mg/L to the drinking water of pregnant CBA mice caused a significant reduction of 18% in the total volume of water consumed by treated mothers over their respective treatment periods (750mg/L control n=15, treated n=17, $t(24)= 5.58$ $p<0.0001$; 1000mg/L control n=4, treated n=5 $t(6)= -3.48$ $p<0.02$). Treated CBA females exposed to the highest dose (1250mg/L) consumed slightly less than controls, although this difference was not significant.

Drinking at these levels means that the approximate average Al intake for CBA females exposed to the 750mg/L dose was 2.0mg over the 7 days. This is similar to the estimated amount of Al taken

in following four days of exposure via i.p. injection. CBA females exposed to the 1000 and 1250mg/L dose of Al for 20 days, consumed an estimated mean total of 7.6mg and 11.6mg elemental Al respectively. This represents a four- and sixfold difference respectively compared to administration by the i.p. route.

As with CBA females, C57 treated mothers drank significantly less water during the treatment period than controls, a difference of 15% in the total water consumed for females exposed to Al at 750mg/L (control n=15, treated n=18, $t(29)= 4.36$ $p=0.0002$). Again, no significant differences existed between control and treated females in their water intake at the highest dose.

The total mean amount of elemental Al ingested over the 7 days by C57 pregnant females was calculated as 2.5mg. The total water consumption was reduced when females were exposed to Al at 1000mg/L (control n=5, treated n=5, t(6) 3.77 $p<0.01$). C57 females in the 1000 and 1250mg/L groups consumed a total mean of 8.7mg and 13.5mg of Al respectively.

C57 mothers exposed to the 1250mg/L dose of Al consumed significantly more food during gestation than controls (control n=8 $\bar{x}=104.22(\pm 3.0)$g, treated n=5 $\bar{x}=117.14(\pm 1.6)$g, $t(9)= -3.79$ $p<0.005$). As the total maternal weight gain and litter size were not influenced by treatment with oral Al, the increased food intake can not be accounted for in these terms. No other differences in food intake were recorded.

Table 6.1: Water intake and estimated amounts of Al ingested by CBA and C57 pregnant females.

Strain		Group	Mean (±S.E.)	Range	Equivalent Al consumed (mg)
CBA	750	Control	40.1 (±1.1)	33.5- 45.4	
		Treated	32.9 (±0.7)	29.5- 38.6	1.8- 2.4
	1000	Control	114.5 (±3.5)	105.0-120.2	
		Treated	94.2 (±4.7)	84.7-111.0	6.9- 9.1
	1250	Control	128.3 (±6.1)	107.0-162.9	
		Treated	114.7 (±3.3)	101.4-119.7	10.3- 12.2
C57	750	Control	48.6 (±1.2)	41.4- 55.9	
		Treated	41.1 (±1.3)	35.4- 51.0	2.2- 3.1
	1000	Control	145.2 (±9.0)	110.7-159.2	
		Treated	106.8 (±4.8)	97.1-124.8	7.9- 10.2
	1250	Control	139.5 (±6.9)	119.0-152.6	
		Treated	132.9 (±9.3)	103.9-157.7	10.6- 16.1

6.3.2 Breeding performance

Administering Al via the oral route did not produce any significant differences in litter size or gestation length in either strain or at any dose.

After cross-fostering, three CBA litters in the 750mg/L group were lost, two from the control and one from the treated group due to neglect or killing of the pups by the mother, which reduced the litter size to less than three. This left four control and five treated litters in this group. No pup mortality occurred in the CBA

1000mg/L (5 control and 4 treated mothers) or 1250mg/L groups (10 control and 10 treated females).

There was a total of 62 control and 63 treated C57 pups at birth within the 750mg/L group. Oral administration of Al to this strain greatly improved the number of mothers who continued to term in comparison with those given i.p. Al (see section **3.9**). However one control litter was lost (on Pd17) and two treated litters, one on Pd10 and the other on Pd17, due to leakage from the water bottles. These losses are reflected in the differing values for degrees of freedom in those measures taken between Pd3 and 21. One control litter and two treated pups were killed in the C57 1000mg/L group by Pd3. Pup mortality in the C57 1250mg/L group also occurred in both experimental groups; two treated and three control females lost pups after cross-fostering.

Litter compositions following cross-fostering for all experimental groups are shown in Table 6.2.

Table 6.2: Litter composition for each strain and each experimental group.

Strain	Treatment mg/L	Cc	Ct	Tt	Tc
CBA	750	8	9	8	10
	1000	8	9	11	8
	1250	20	19	20	20
C57BL/6J	750	14	13	14	15
	1000	10	9	9	9
	1250	13	6	10	12

6.3.3 Maternal weight

CBA mothers exposed to 1000mg/L gained more weight during Gd10 to 18 than controls (control n=7 $\bar{x}$=7.79(±1.08)g, treated n=5 $\bar{x}$=9.2(±0.86)g), although this difference was not significant. Postnatal CBA maternal weight was not influenced by prenatal exposure to oral Al. Similarly, C57 maternal weight gain during gestation and the suckling period was largely unaffected by oral exposure to Al.

6.3.4 Maternal behaviour

At the 750mg/L dose Mann-Whitney U-tests revealed only slight differences between control and Al-treated CBA mothers in the pattern of maternal behaviour. Control CBA mothers exhibited an increased frequency of *nursing* on Pd2 (control median=5.5, treated median=0, U=20.0 $p<0.02$), and an increase in the number of bouts of *licking* which were of longer duration than those of Al-exposed CBA females on Pd2 (frequency control median=5.5, treated median=0, U=20.0 $p<0.02$; duration control median=234.9s, treated median=0.0, U=20.0 $p<0.02$). Al-treated mothers had a greater frequency and duration of *nest-building* behaviour than control mothers on Pd7, although this difference was only marginally significant. On Pd14 control females spent less time involved in *active* behaviour (control median=120.0s, treated median=457.7s, U=1.0 $p<0.05$).

As with CBA mothers, the pattern of maternal behaviour of control and treated C57 mothers was largely unaffected by exposure

to the 750mg/L dose. Table 6.3 summarises the only differences between C57 control and treated dams all of which occurred on Pd10. It can be seen from this that C57 control mothers were more involved in *nest-building* and in non pup-directed maternal activities than treated mothers on this day.

Table 6.3: Effect of gestational exposure to oral Al (750mg/L) on C57 maternal behaviours on postnatal day 10.

KEY: C=control T=treated

Maternal Behaviour		Median	U-value	p value
NESTBUILD				
Duration	C	25.8	47.0	=0.02
	T	0.0		
Frequency	C	3.0	48.5	<0.02
	T	0.0		
ACTIVE				
Duration	C	270.3	50.5	<0.009
	T	34.3		
Frequency	C	16.5	46.5	=0.03
	T	6.0		
REAR				
Duration	C	72.8	46.0	<0.04
	T	19.1		
Frequency	C	15.5	49.5	<0.02
	T	5.0		
GROOM				
Frequency	C	3.0	46.5	<0.03
	T	1.0		

Maternal behaviour observations were not undertaken at the 1000mg/L dose. Only those maternal behaviours for which group differences were found at the 1250mg/L dose are described below.

Nursing - In general, CBA control dams (n=10) exhibited a greater number of bouts of *nursing* than treated mothers (n=10). This difference was significant only on Pd3 (control median=2.5, treated median=1.0, U=86.0 $p<0.005$).

C57 treated mothers (n=5) spent little time involved in *nursing* until Pd7. On Pd2 control mothers (n=7) spent significantly more time *nursing* compared to treated females (control median=245.0s, treated median=0.0, U=31.0 $p<0.03$). In contrast to control C57 mothers, who exhibited the expected decline in *nursing* with time, treated C57 females increased the amount of time spent in this behaviour as the pups got older.

The frequency of *nursing* behaviour was lower in treated females on Pd2, although this difference was marginally significant on Pd2 but reached significance on Pd3 (control median=2.0, treated median=0.0, U=19.0 $p<0.03$); control mothers exhibited an increased number of bouts.

Licking - Although the occurrence of *licking* behaviour was rare in both strains, control C57 mothers showed a greater frequency (control median=2.0, treated median=0.0, U=30.5 $p=0.03$) and duration (control median=104.2s, treated median=0.0, U=32.0 $p<0.02$) of bouts of this behaviour on Pd2. There were no group differences within the CBA strain.

Grooming - Treated CBA females spent more time *self-grooming* during the first five test days but this effect was reversed on Pd10, when CBA controls spent significantly more time *grooming* than treated mothers (control median=173.3s, treated median=90.9s, U=84.0 p=0.01).

Eating and Drinking - The duration of bouts of *eating and drinking* was greater for treated CBA females during the first postnatal week, a difference which was marginally significant on Pd3. Infact, CBA control females were not observed in these behaviours until Pd7, and on Pd10 the difference was reversed (control median=317.8s, treated median=35.5s, U=79.0 p<0.03). There was also a significant difference in the frequency of this behaviour on Pd10 (control median=3.5, treated median=1.5, U=84.5 p<0.009).

6.3.5 Pup Weight

In utero exposure to oral Al resulted in a significantly lower birth weight within the treated CBA 750mg/L group only (control n=29 $\bar{x}$=1.36($\pm$0.02)g, treated n=49 $\bar{x}$=1.28($\pm$0.02)g, $t(75)$= 2.97 p=0.004), with control pups weighing 6% more at birth. Although CBA control pups weighed more at birth than pups treated at a dose of 1000mg/L, this difference was not significant (control n=26 $\bar{x}$=1.4($\pm$0.02)g, treated n=29 $\bar{x}$=1.36($\pm$0.03)g). At the 750 and 1000mg/L doses, there were no effects of prenatal or foster mother treatment on CBA pup growth up to weaning. Thus, the lower weight of the treated pups in the 750 and 1000mg/L groups did not persist beyond birth.

CBA pups exposed to the 1250mg/L dose had comparable weights at birth to controls. However, there was a marginally significant effect of foster mother treatment on CBA pup weight up to weaning, pups reared by treated mothers were heavier than pups fostered to control mothers.

C57 birth weights were unaffected by prenatal exposure to oral Al either at the 750 or 1250mg/L doses. There was only a small sample of pups from the 1250mg/L dose and this may explain why no significant effect could be detected. However, Al-treated 1000mg/L C57 pups weighed 5% less than controls at birth (control n=46 $\bar{x}$=1.38($\pm$0.02)g, treated n=30 $\bar{x}$=1.31($\pm$0.02)g, $t(55)$= 2.15 $p<0.04$).

There was a highly significant effect of foster mother treatment on C57 pup body weight during the preweaning period when pups were exposed to Al at the lowest dose (750mg/L) ($F(1,36)=5.68$ $p<0.03$); pups reared by Al-exposed females weighed more than pups fostered to control females (see Fig. 6.1). The divergence in pup weights began as early as Pd3 and increased with age. From Fig. 6.1a **Ct** pups were the heaviest and **Cc** the lightest group, a difference of 6% by Pd21. Although the **Tt** group weighed more the **Tc** group this difference was only slight.

There were no significant differences in C57 pup body weight following exposure to Al at a dose of 1000mg/L. However, there was a significant effect of foster mother treatment on pup weight when Al was administered at 1250mg/L, interestingly, in this case pups fostered to control mothers weighed more than those reared by treated mother ($F(1,33)=9.71$ $p<0.004$, see Fig. 6.1b). The

Fig. 6.1a *750 mg/L*

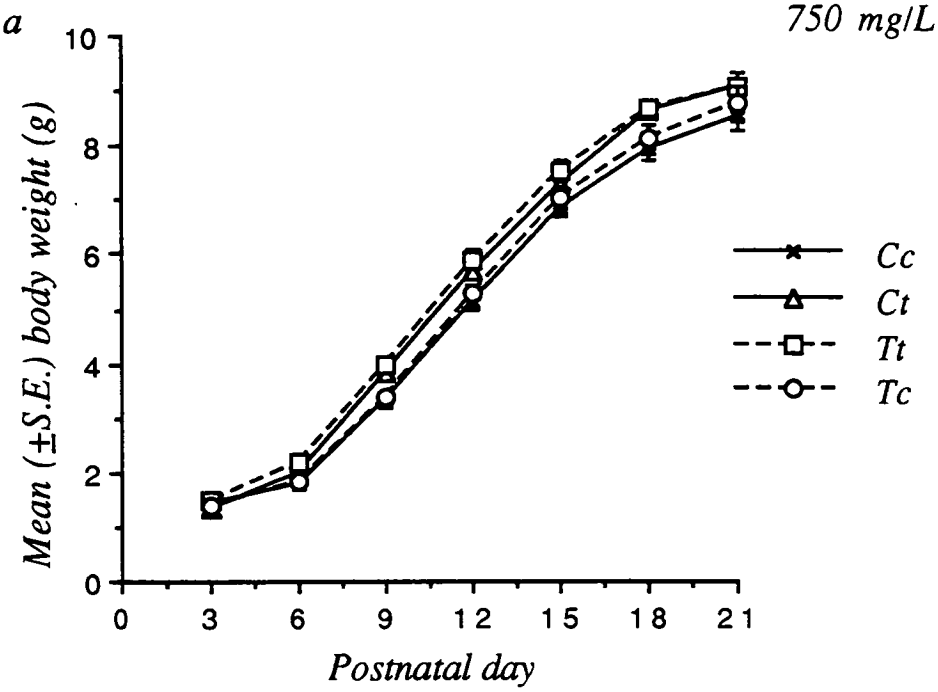


Fig. 6.1b *1250mg/L*

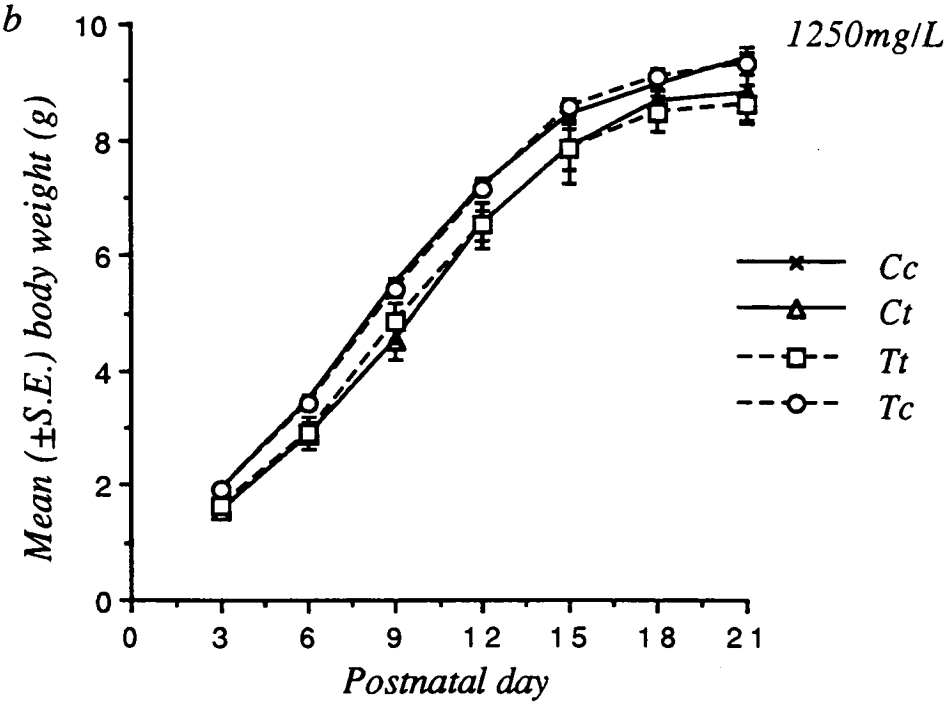


Fig. 6.1: Effect of prenatal exposure to oral aluminium sulphate on C57BL/6J pup body weight.

difference between **Cc** and **Ct** pups was greatest on Pd9 (17.6%) and on Pd3 for **Tt** and **Tc** pups (15.9%).

6.3.6 Adult weight

Separate ANOVA's of CBA adult male and female body weights did not reveal any significant differences between experimental groups at the 750 or 1000mg/L doses of oral Al.

At the lowest dose there was a marginally significant effect of foster mother treatment on C57 adult male weights, the increase in body weight of animals reared by treated mothers persisted into adulthood. This was also true for C57 adult females as ANOVA revealed a significant interaction between week and foster mother treatment ($F(12,156)=2.57$ $p<0.04$), the divergence in female weight was not clearly evident until after 13 weeks of age.

At the 1000mg/L dose there was a significant interactive effect of week, prenatal treatment and foster mother treatment on C57 adult female weights ($F(11,176)=2.2$ $p=0.05$). C57 adult male weights were unaffected by treatment at this dose.

Subjects exposed to the 1250mg/L dose were not followed up into adulthood.

6.3.7 Ultrasonic calling

Unlike the effects of i.p. Al, the total number of ultrasonic calls emitted by CBA pups was largely unaffected by oral exposure to Al at 1000mg/L. Treated pups produced a low constant level of calling throughout the test period compared to **Cc** pups (see Fig.

Fig. 6.2a

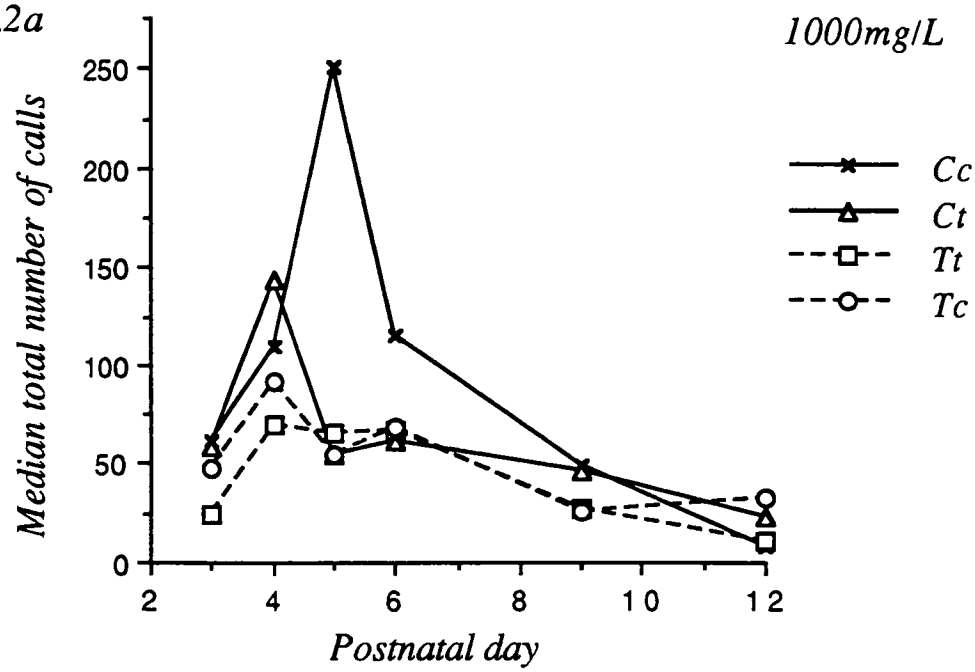


Fig. 6.2b

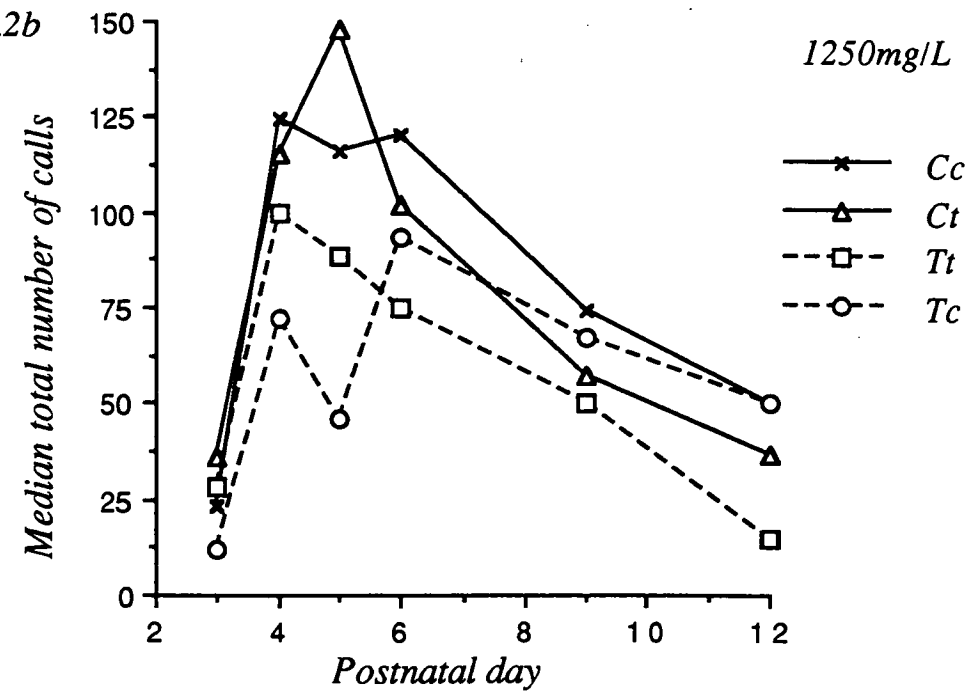


Fig. 6.2: The total number of calls emitted by CBA pups in a five minute test following prenatal exposure to oral aluminium sulphate at a dose of (a) 1000mg/L and (b) 1250mg/L.

6.2a). Similarly, output from **Ct** pups was constant but at a higher level than treated pups. **Cc** pups exhibited an increase in calling to a peak on Pd5 followed by a subsequent decline. Although **Ct**, **Tt** and **Tc** pups seemed to peak on Pd4, a day before the **Cc** group, it is unlikely that this implies a difference in the achievement of thermoregulation as their overall production of ultrasounds followed a fairly constant pattern. On Pd5 there was a significant group difference in calling ($H(3)=8.68$ $p<0.04$), with **Cc** pups having a 5-fold difference in calling rate compared to the other three groups.

Similarly, at 1250mg/L, CBA control pups called more than treated pups, a difference which was marginally significant on Pd4. **Cc** and **Ct** pups reached peak calling on Pd4 (125 and 119 calls/5 min respectively), whilst **Tt** pups peaked on Pd5 (97 calls/5 min) and the **Tc** group on Pd6 (92 calls/5 min), two days later than control pups (see Fig. 6.2b).

Turning to the C57 strain, we can observe from Fig. 6.3a that **Cc** pups called more than the other groups following exposure at the 1000mg/L dose. However, it is difficult to pick up any clear differences resulting from prenatal treatment as the range of calling is so narrow. Conversely, following exposure to Al at 1250mg/L, there is a trend towards treated C57 pups calling more than controls. Pups from all experimental groups produced a maximum number of calls on Pd5 (see Fig. 6.3b), followed by a sharp decline.

Fig. 6.3a

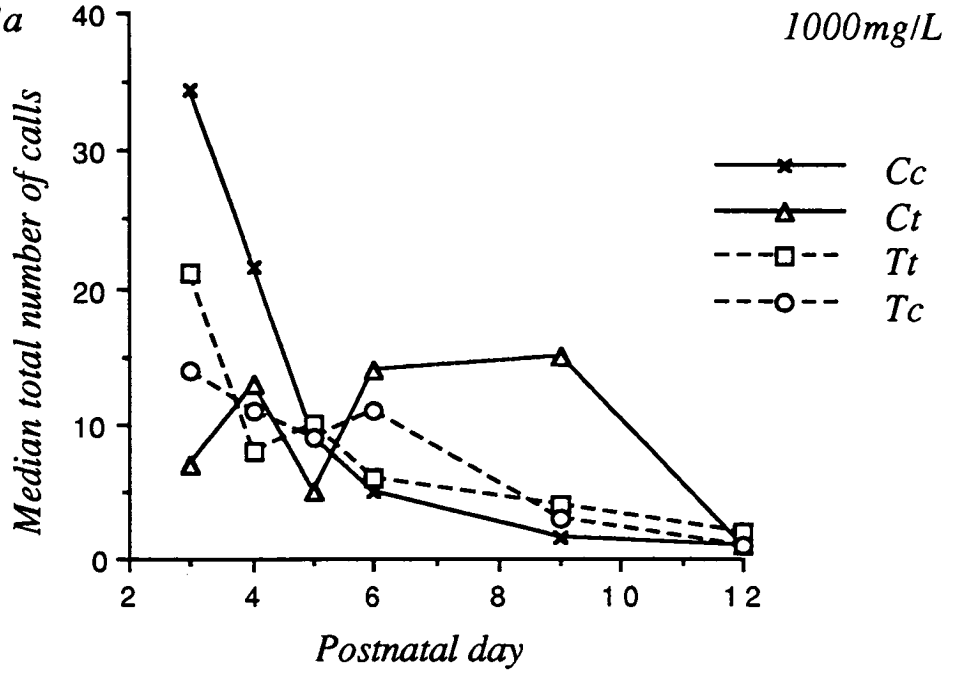


Fig. 6.3b

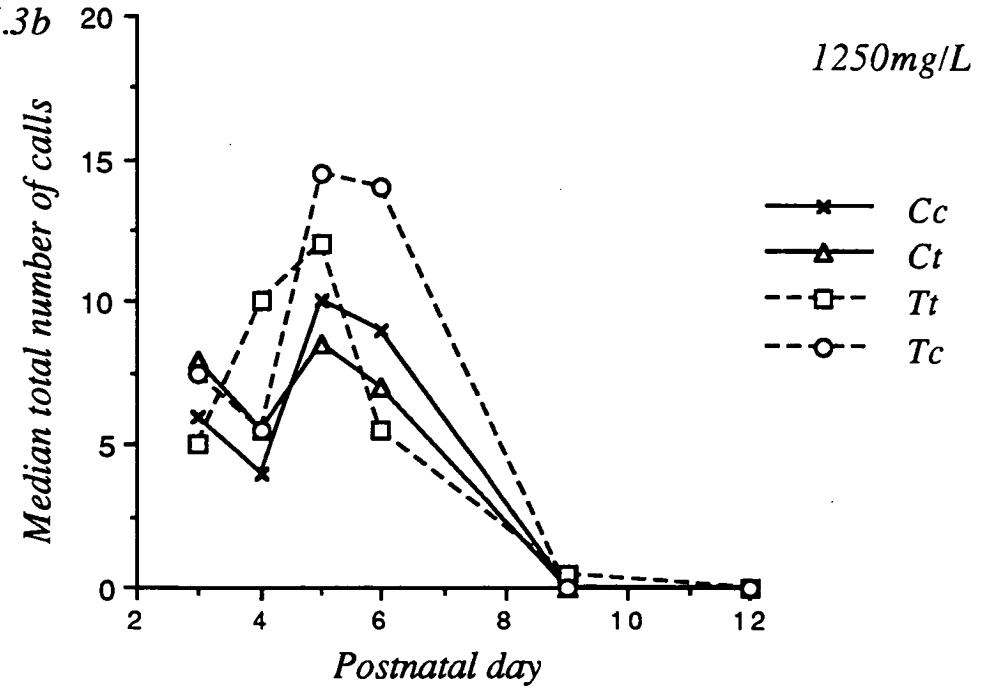


Fig. 6.3: The total number of calls emitted by C57BL/6J pups in a five minute test following prenatal exposure to oral aluminium sulphate at a dose of (a) 1000mg/L and (b) 1250mg/L.

6.3.8 Fox tests

The percentage of pups judged as having acquired a mature response in the Fox tests did not differ significantly between the 750mg/L CBA experimental groups. At the 1000mg/L dose the only difference occurred in *swift righting* on Pd12; more CBA pups fostered to treated mothers had acquired a mature response by this day ($X^2(3)=14.924$ $0.01>p>0.001$).

There was a significant group difference in the number of CBA 1250mg/L pups reaching an adult response in *forelimb grasping* on Pd12 ($X^2(3)=16.466$ $p<0.001$), pups fostered to treated females performed better than those reared by control mothers. At this dose the performance of **Cc** pups was correlated with body weight ($r=-0.636$ $0.005>p>0.002$). There was a maternal effect on performance in *cliff aversion* on Pd9 ($X^2(3)=9.131$ $0.05>p>0.02$), a greater number of pups fostered to control mothers achieved an adult response on this test. Fox test score was correlated with weight for **Cc** and **Tc** pups ($r=-0.542$ $0.02>p>0.01$; $r=-0.85$ $p<0.001$ respectively).

Treated 750mg/L C57 pups performed better in the *slow righting* test on Pd6 ($X^2(3)=13.6$, $0.01<p>0.001$), and in *eye opening* on Pd15 ($X^2(3)=14.56$, $0.01<p>0.001$). **Tt** pup scores in both these measures were correlated with body weight (*slow righting* $n=12$ $r=0.697$ $0.02<p>0.01$; *eyes open* $r=0.697$ $0.02>p>0.01$) and **Tc** scores in *eyes open* ($n=15$ $r=0.756$ $0.002>p>0.001$).

6.3.9 Pup behaviours

Pup behaviours were observed following the 750mg/L treatment only. There were no significant prenatal, maternal or sex effects on the frequency or duration of any of the pup behaviours recorded for the CBA strain. However, C57 pups showed significant group differences in the following pup behaviours:-

Righting - Kruskal-Wallis one-way ANOVA revealed a significant group difference in the duration of time spent *righting* on Pd3 ($H(3)=8.9$ $p=0.03$), with treated pups spending more time in this behaviour. The difference between **Cc** and **Tt** pups was significant at $p<0.05$.

Crawling - There was a significant difference between experimental groups in the frequency ($H(3)=9.58$ $p<0.03$) and duration ($H(3)=7.96$ $p<0.05$) of bouts of *crawling* on Pd6. In both cases the difference between **Ct** and **Tt** pups was significant at $p<0.05$, **Ct** pups being more involved in this behaviour.

Square crossing - ANOVA revealed a significant main effect of foster mother treatment on pup *activity* between Pd9-18 as shown in Fig. 6.4a ($F(1,38)=4.67$ $p<0.04$); pups fostered to treated mothers crossed more squares than pups raised by control mothers. Activity levels in all groups increased with age.

Headup - There was a group difference in the frequency of bouts of *headup* behaviour which was marginally significant on Pd3, but

Fig. 6.4a

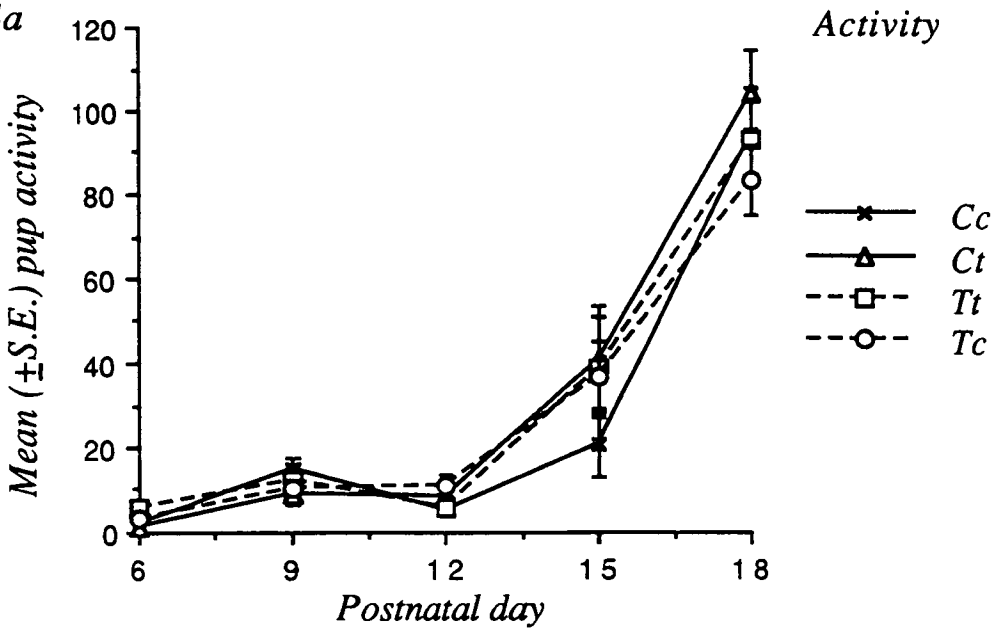


Fig. 6.4b

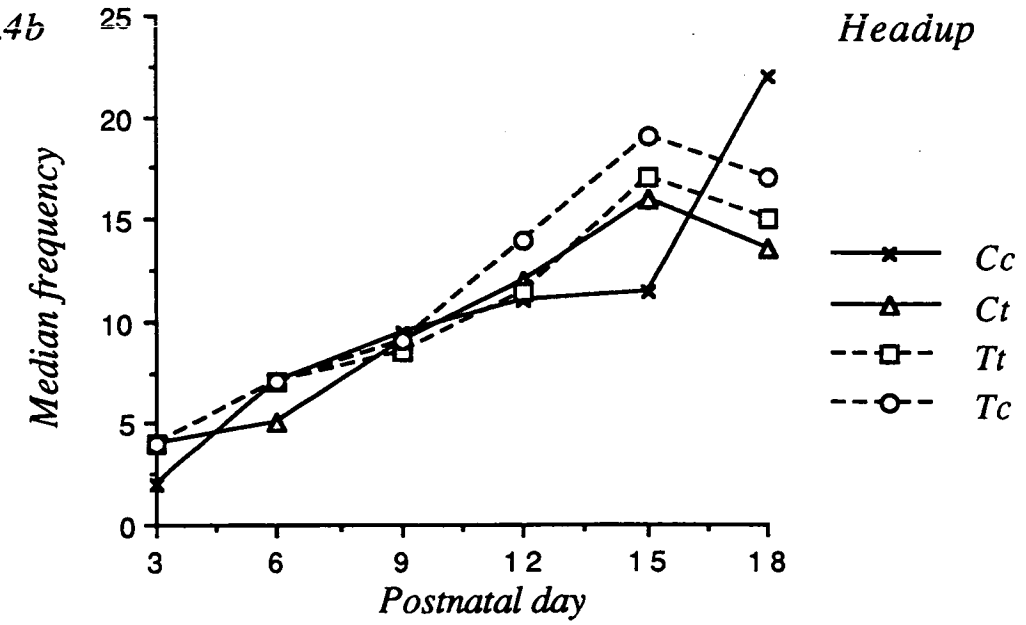


Fig. 6.4: Effect of prenatal exposure to oral aluminium sulphate (750mg/L, Gd10-16) on C57BL/6J pup (a) activity and (b) headup behaviour.

reached significance on Pd6 ($H(3)=8.92$ $p=0.03$) and on Pd18 ($H(3)=7.78$ $p=0.05$), as shown in Fig. 6.4b.

Edgeon - There was a significant group difference in the duration ($H(3)=8.51$ $p<0.04$) and frequency ($H(3)=9.24$ $p<0.03$) of *edgeon* behaviour on Pd15. In the latter case the difference between **Cc** and **Tc** scores was significant at $p<0.05$.

6.3.10 Activity at weaning

The number of squares crossed by CBA pups was unaffected by treatment with 750mg/L oral Al. Conversely, when the dose was increased to 1000mg/L there was a significant effect of foster mother treatment on CBA activity ($F(1,28)=5.58$ $p<0.03$), pups fostered to Al-treated females exhibited lower activity levels (see Fig. 6.5). At the 1250mg/L dose there was a significant interaction between prenatal treatment and sex ($F(1,67)=4.21$ $p<0.05$) on CBA pup activity and between minute of test and sex ($F(4,268)=2.45$ $p=0.05$).

The activity of C57 pups was unaffected by exposure to Al at a dose of 750mg/L. Although C57 **Cc** pups in the 1000mg/L group exhibited lower activity scores during minutes 1 and 2 than pups in the other groups, this difference was not significant. There was a significant interactive effect of all variables on C57 1250mg/L pup activity at weaning ($F(4,132)=2.92$ $p<0.04$), no other interactions were significant. There were no differences in activity levels following prenatal exposure of C57 pups to Al at a dose of 1250mg/L.

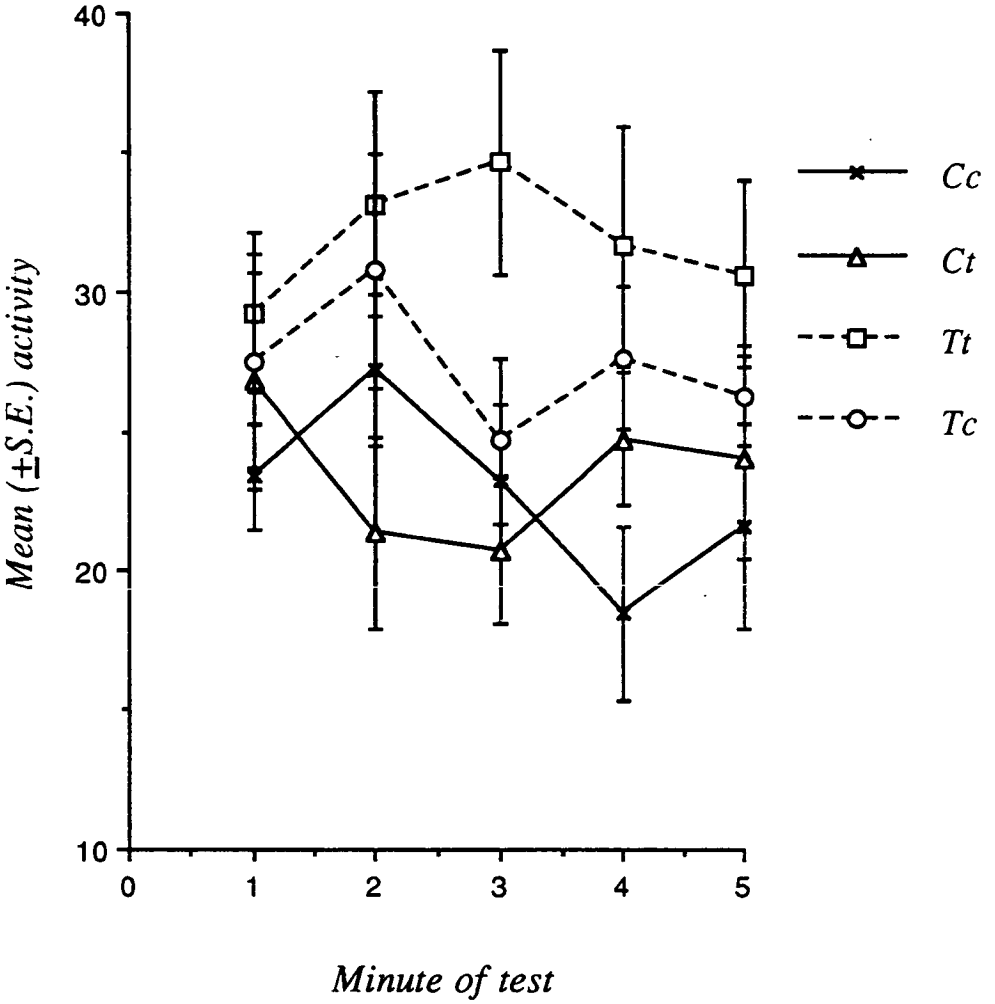


Fig. 6.5: Effect of prenatal exposure to oral aluminium sulphate (1000mg/L, Gd1-20) on the number of squares crossed by CBA pups in a five minute openfield test.

CBA 750mg/L **Tt** pups took the least (median=26.0s) and **Tc** pups the longest time (median=62.5s) to contact the novel object placed in the centre of the openfield at the end of the activity test. Conversely, control C57 pups took longer to make contact than treated pups (median=47.0, 53.0, 26.0 and 29.0s for **Cc, Ct, Tt** and **Tc** pups respectively). These differences were not significant.

There were no differences in latencies between experimental groups at the 1000mg/L dose of Al in either strain.

At the 1250mg/L dose, CBA **Cc** pups required less time than the other groups to make contact whilst there was no difference in the time taken by C57 pups. There is a clear strain difference in approach time at this dose, C57 pups took less time to make contact than CBAs.

6.3.11 Maze test

There were problems with these learning experiments because within both CBA 750mg/L control (5 out of 10) and treated groups (4 out of 8) only half of the subjects reached the criterion of two errorless trials. Treated subjects required more days to reach criterion than controls (control median=12.0, treated median=16.5 days). Control and treated subjects did not differ in any other measure recorded. Even fewer animals reached criterion during 14 days of testing when the dose was increased to 1000mg/L (control 1 out of 7, treated 5 out of 9). As few CBA animals completed the task the scopolamine challenge was not undertaken.

54% (7 out of 13) of C57 750mg/L control and 71% (10 out of 14) treated subjects reached criterion of two consecutive errorless

trials in the maze. In addition, treated males needed less days ($\bar{x}$=7.9(±0.89)days) to acquire the maze than controls ($\bar{x}$=8.86(±1.3)days); chi-square tests showed that neither of these measures were significant. ANOVA did not reveal any group differences in choice accuracy between the experimental groups. As there were not enough **Ct** pups to challenge with scopolamine, analysis did not take foster mother treatment into consideration. There was a highly significant effect of scopolamine on the number of trials needed to enter all 8 arms ($F(1,11)=7.95$ $p<0.02$); scopolamine produced the expected decrease in choice accuracy, both control and treated animals required more trials. There was also a significant interaction between day and prenatal treatment ($F(2,22)=7.14$ $p<0.009$), treated animals took fewer trials to enter all 8 arms, and between these variables and the challenge ($F(2,22)=5.66$ $p<0.02$), by the third day of treatment with scopolamine treated animals no longer showed any impairment (see Fig. 6.6a). Treatment with scopolamine also produced a significant reduction in the number of correct responses made by both groups ($F(1,11)=14.45$ $p<0.003$). Al-treated subjects made slightly more correct responses than controls (see Fig. 6.6b).

The number of arms in the radial maze was reduced to six for C57 1000mg/L males in an attempt to improve performance. 62.5% of control and 57.1% treated animals completed the maze test, treated subjects requiring slightly longer to do so (control median=8.0, treated median=8.5 days). Although there were slight differences in the number of trials taken by control animals to enter all 6 arms, they did not reach significant levels.

Fig. 6.6a

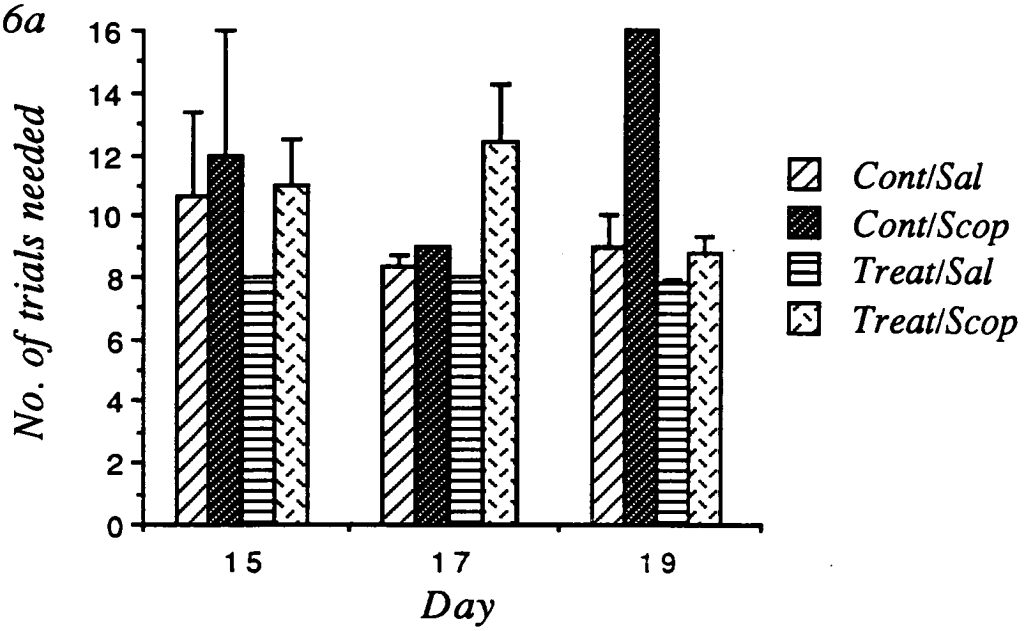


Fig. 6.6b

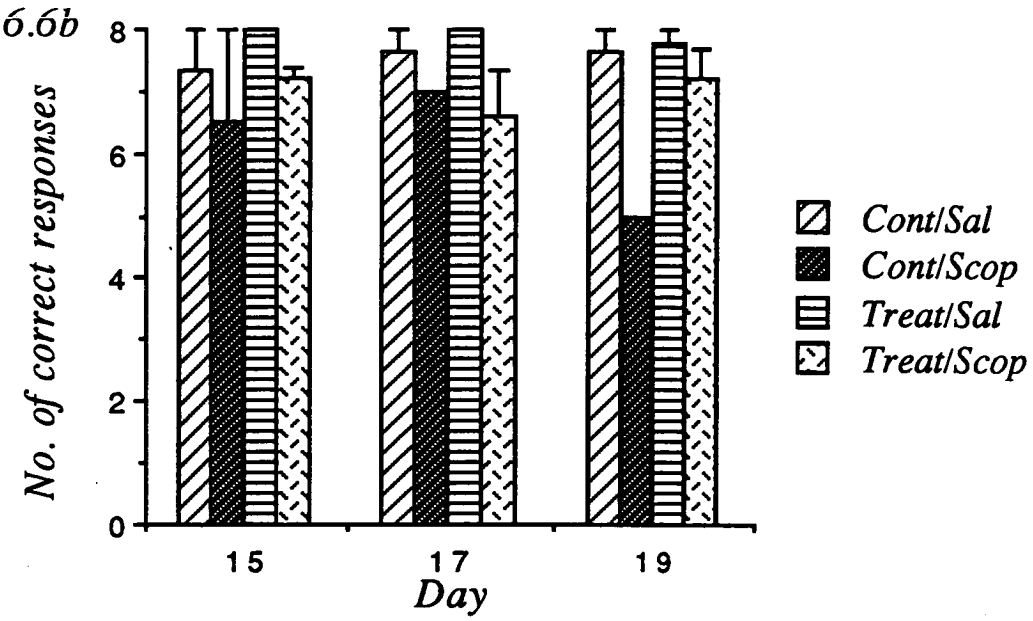


Fig. 6.6: Effect of a scopolamine challenge on C57BL/6J maze performance.

6.3.12 Adult activity tests

CBA adult female activity at 22 weeks of age was unaffected by oral Al at 750 or 1000mg/L. This was also the case for C57 females exposed to Al at the lowest dose. However, at the 1000mg/L dose of Al, there was a significant interactive effect of minute of test, prenatal treatment and foster mother treatment on C57 adult female activity ($F(4,68)=4.23$ $p<0.009$); **Ct** females were more active than **Tcs** during minutes 3-5. On day 4 of the test there was a significant main effect of prenatal treatment on activity ($F(1,17)=6.19$ $p<0.03$), control animals were more active than treated subjects. This was reflected in the mean total squares crossed which was 222.5, 234.4, 174.2 and 182.3 for **Cc**, **Ct**, **Tt** and **Tc** animals respectively.

6.3.13 ChAT Activity

Tables 6.4 to 6.7 summarise the levels of ChAT activity in the different brain regions at 23 weeks of age, for male and female animals of both strains whose mothers were exposed to oral Al at a dose of 1000mg/L or saline during gestation. ChAT activity was assayed at one age only due to the moderate sample sizes. Each measure is the mean (±S.E) and represents pooled brains of 3-4 animals in each group.

The level of ChAT activity differed between male and female CBA animals in all brain regions considered, and tended to be increased in female brains. This was also the case for females

within the C57 strain although not all brain regions e.g. the cerebral cortex, showed a sex-dependent difference in enzyme activity.

The strain dependent differences in ChAT levels were not as pronounced as those arising from gender. However, CBA females exhibited consistently higher levels of ChAT than C57 females except in the hypothalamus. This tended to be true for CBA male subjects except in the case of the hippocampus and brainstem where ChAT levels were greater in C57 males.

Table 6.4 shows the levels of ChAT activity within 7 brain regions for CBA male subjects, no data is available for the hypothalamus. **Ct** animals had similar or lower levels of ChAT than **Ccs** except in the cerebellum where there was a 36% increase. This decrease in activity can also be seen within the **Tt** group except in the striatum, although the increase in **Tt** levels was only slight compared to **Cc** males (8%) in this region. This trend of exposure to oral Al reducing ChAT levels followed for **Tc** males. However, as with the **Tt** group, this was not the case within the striatum where there was a marked increase (41%) in ChAT levels compared to **Cc** males. Thus, the overall effect of exposure to Al, either pre- or postnatally, on CBA males was a reduction in ChAT activity.

The levels of ChAT activity in each of the 8 brain regions for CBA females are shown in table 6.5. **Ct** females exhibited a lower ChAT level compared to **Cc** females except in the hypothalamus, striatum and brainstem. The greatest difference occurred in the hypothalamus (65%), although the value for activity in the **Cc** group seems remarkably low. With the exception of the brainstem, **Tt** females tended towards an increase in ChAT activity compared to **Ccs**. **Tc** females exhibited consistently lower levels

Brain region	Cc ♂	Ct ♂	Tt ♂	Tc ♂	Prenatal Treatment	Maternal Treatment	Interaction
Cerebral cortex	490.5 ± 11.9	501.9 ± 7.5	402.7 ± 5.6	357.5 ± 2.7	p<0.0001	p<0.002	p<0.04
Cerebellum	65.5 ± 1.5	101.6 ± 2.2	49.5 ± 1.2	48.6 ± 1.0	p<0.0001	p<0.0001	p<0.0001
Hippocampus	617.4 ± 7.8	503.9 ± 9.2	581.4 ± 10.5	678.1 ± 15.7	p<0.0001	p<0.0001	N.S.
Midbrain	791.0 ± 14.9	499.8 ± 8.2	565.6 ± 17.6	573.6 ± 17.9	p=0.0001	p<0.0001	p<0.0001
Hypothalamus	N.D.	N.D.	N.D.	N.D.	-	-	-
Nucleus magnocellularis	1683.4 ± 37.6	945.6 ± 26.4	1372.6 ± 18.4	1399.1± 53.5	N.S.	p<0.0001	p<0.0001
Straitum	1213.6 ± 4.7	1029.2 ± 17.5	1320.2 ± 18.5	2044.1 ± 46.2	p<0.0001	p<0.0001	p<0.0001
Brainstem	739.8 ± 11.2	719.9 ± 11.7	555.9 ± 10.0	411.1 ± 5.3	p<0.0001	p<0.0001	p<0.0001

Table 6.4: Effect of oral exposure to aluminium sulphate (1000mg/L) throughout gestation on the level of ChAT activity in different brain regions in 23-week old CBA/T6 male subjects. N.D.= Not Determined

Brain region	Cc ♀	Ct ♀	Tt ♀	Tc ♀	Prenatal Treatment	Maternal Treatment	Interaction
Cerebral cortex	560.2 ± 32.6	452.1 ± 14.0	884.1 ± 13.6	383.7 ± 5.7	p<0.0001	p<0.0001	p<0.0001
Cerebellum	102.2 ± 2.9	79.4 ± 12.8	42.0 ± 2.3	77.4 ± 2.8	p=0.0002	p=0.0004	N.S.
Hippocampus	994.5 ± 69.6	699.4 ± 25.0	1061.9 ± 19.7	759.8 ± 11.8	N.S.	N.S.	p<0.0001
Midbrain	737.2 ± 13.3	644.6 ± 32.8	911.6 ± 23.5	591.0 ± 15.9	p<0.02	p=0.0001	p<0.0001
Hypothalamus	245.5 ± 7.6	702.1± 17.4	570.7 ± 13.3	768.9 ± 19.2	p<0.0001	p<0.0001	p<0.0001
Nucleus magnocellularis	1703.7± 37.2	1718.4 ± 26.3	2007.6 ± 60.7	793.8 ± 17.5	p<0.0001	p<0.0001	p<0.0001
Straitum	1536.8 ± 19.2	1987.9 ± 67.8	1487.7 ± 18.4	802.3 ± 26.6	p<0.0001	p<0.0001	p<0.007
Brainstem	788.9 ± 16.7	969.5 ± 10.7	690.8 ± 24.1	276.6 ± 5.1	p<0.0001	p<0.0001	p<0.0001

Table 6.5: Effect of oral exposure to aluminium sulphate (1000mg/L) throughout gestation on the level of ChAT activity in different brain regions in 23-week old CBA/T6 female subjects.

than **Cc** females but again this was not the case in the hypothalamus. Thus, within CBA females, postnatal exposure to Al (**Ct** and **Tt** subjects) seemed to cause an increase in ChAT activity.

C57 males in the **Ct** group (see Table 6.6) exhibited both increases (within the midbrain, hypothalamus, nucleus and striatum) and decreases (cerebral cortex, hippocampus and brainstem) in ChAT levels compared to **Cc** males. The largest increase occurred in the hypothalamus (46%) and the greatest decrease in activity within the brainstem (43%). With the exception of the brainstem, **Tt** males showed consistent increases in ChAT activity compared to **Cc** males. This increase was particularly marked in the nucleus (40%). Again, within the **Tc** group the effect of Al varied with brain region. Increases in ChAT were recorded in the cerebellum, hypothalamus and striatum, whilst decreases were found in the cerebral cortex and brainstem.

As with C57 males, C57 females in the **Ct** group showed both increases and decreases in ChAT activity depending on brain region (see Table 6.7). There was a marked increase in ChAT activity in the nucleus magnocellularis of **Tt** females, a difference of 25%, but the other brain regions tended towards a decrease in activity. **Tc** females also exhibited this trend of a decrease in activity compared to **Cc** females except for a slight increase in activity within the brainstem (6%). Again, apart from inconsistencies within the **Ct** group, C57 females showed a general trend towards a decrease in ChAT levels following exposure to Al, which is in direct contrast to CBA females.

Brain region	Cc ♂	Ct ♂	Tt ♂	Tc ♂	Prenatal Treatment	Maternal Treatment	Interaction
Cerebral cortex	433.8 ± 25.3	402.5 ± 4.8	426.4 ± 7.5	416.8 ± 8.6	N.S.	N.S.	N.S.
Cerebellum	58.9 ± 1.9	63.7 ± 1.8	77.9 ± 2.2	129.4 ± 2.5	p<0.0001	p<0.0001	p<0.0001
Hippocampus	641.0 ± 8.8	567.1 ± 8.5	950.4 ± 15.6	650.0 ± 6.1	p<0.0001	p<0.0001	p<0.0001
Midbrain	714.1 ± 29.2	885.3 ± 15.7	829.6 ± 13.8	695.2 ± 12.6	N.S.	p<0.0001	N.S.
Hypothalamus	467.9 ± 59.3	871.7 ± 7.0	627.2 ± 9.5	623.6 ± 15.8	N.S.	p<0.0001	p<0.0001
Nucleus magnocellularis	1276.2 ± 11.7	1866.6 ± 17.9	2133.3 ± 72.1	1215.4 ± 9.6	p<0.02	p<0.0001	p=0.0003
Straitum	918.0 ± 11.3	1547.4 ± 33.9	1312.8 ± 24.9	1891.3 ± 18.0	p<0.0001	N.S.	p<0.0001
Brainstem	857.3 ± 12.8	489.8 ± 5.5	427.3 ± 5.4	564.8 ± 9.1	p<0.0001	p<0.0001	p<0.0001

Table 6.6: Effect of oral exposure to aluminium sulphate (1000mg/L) throughout gestation on the level of ChAT activity in different brain regions in 23-week old C57BL/6J male subjects.

Brain region	Cc ♀	Ct ♀	Tt ♀	Tc ♀	Prenatal Treatment	Maternal Treatment	Interaction
Cerebral cortex	383.6 ±7.6	485.9 ±10.2	354.3 ±3.1	264.8 ±6.0	p<0.0001	p<0.0001	N.S.
Cerebellum	95.5 ±2.5	85.2 ±1.5	62.7 ±1.5	91.3 ±3.4	p<0.0001	p<0.0001	p<0.001
Hippocampus	774.4 ±4.4	778.8 ±9.5	657.2 ±16.9	646.7 ±18.2	p<0.0001	N.S.	N.S.
Midbrain	605.1 ±10.3	705.5 ±19.7	544.3 ±39.5	469.2 ± 7.8	p<0.0001	p<0.002	N.S.
Hypothalamus	661.9 ±43.5	522.6 ±8.2	503.1 ± 9.3	554.1 ±10.7	p<0.02	p<0.001	N.S.
Nucleus magnocellularis	1511.7 ±23.6	1083.4 ±9.2	2008.8 ±29.5	1049.2 ±18.8	p<0.0001	p<0.0001	p<0.0001
Straitum	1023.9 ±34.6	823.0 ±7.9	947.6 ±14.5	780.3 ±16.9	p<0.05	N.S	p<0.0001
Brainstem	528.0 ± 5.1	642.3 ±3.6	607.8 ±17.9	561.9 ±12.0	N.S.	p<0.0001	p<0.007

Table 6.7: Effect of oral exposure to aluminium sulphate (1000mg/L) throughout gestation on the level of ChAT activity in different brain regions in 23-week old C57BL/6J female subjects.

6.4 DISCUSSION

The experiments described in this chapter investigated the effects of oral exposure, via the dam's drinking water, of pregnant CBA and C57 females to $Al_2(SO_4)_3$ at three different doses (750, 1000 and 1250mg/L). There are many complexities and inconsistencies with the results, but the following trends have emerged.

Adding Al to the drinking water at a dose of 750 and 1000mg/L reduced the amount consumed compared to controls. As control water was acidified to the same pH as the Al solution, this can not be explained in terms of increased acidity. Interestingly, the reduction in water intake was not as pronounced at the highest dose.

Exposure to oral Al, at any of the doses employed, did not result in maternal weight loss or a reduction in weight gain during the treatment period. This is in agreement with previous studies in mice (Golub, *et al.*, 1987) and in rats (Bernuzzi, *et al.*, 1986, 1989; Muller, *et al.*, 1990). On the other hand, Paternain, *et al.*, (1988) have reported a decrease in maternal weight gain following exposure of Sprague-Dawley rats to $Al(NO_3)_3$ at a dose of 180, 360 or 720mg/L during days 6-14 of gestation. The lack of effect of oral Al on maternal weight during gestation is in contrast to the maternal weight data following exposure by the i.p. route (see section *3.4.3*); Al-treated females gained less weight during days 10 to 18 of gestation compared to controls. This suggests that the reduction in weight gain may have resulted from the combination of the stress of injection with being exposed to Al.

Similarly, oral Al did not alter breeding performance or pup mortality within either mouse strain. However, a number of studies have found a significant increase in postnatal pup mortality (Bernuzzi, *et al.*, 1986, 1989; Domingo, *et al.*, 1987a; Domingo, *et al.*, 1987b) following oral exposure to $AlCl_3$ or $Al(NO_3)_3$.

Slight differences in maternal behaviour resulted from exposure to Al at the 750mg/L dose. CBA females exhibited increased frequencies of pup-directed maternal activities and a decreased duration of *active* behaviour. Conversely, C57 control females spent more time *nest-building* and in non pup-directed activities. At the higher dose (1250mg/L) only moderate differences between control and treated mothers were observed. In both strains control females exhibited more *nursing* behaviour compared to treated mothers, which declined with age in the CBA strain, but increased as a function of age for C57 treated mothers. Thus, within the C57 strain exposure to Al prolonged the expression of *nursing* behaviour. With respect to *self-grooming* and *eating and drinking*, CBA treated mothers showed an increased involvement in these behaviours during the first postnatal week, but by Pd10 this trend was reversed.

The effect of oral Al on pup body weight was both strain and dose dependent. CBA pups treated at the 750mg/L dose had a lower weight at birth than controls, but this may have resulted from the slight increase in litter size in this group rather than from a direct effect of exposure to Al. CBA pup body weight during the preweaning period was unaffected by exposure to Al at the 750mg/L dose. In fact, there were no significant differences in any of the physical landmarks or behavioural measures investigated at the

750mg/L dose within the CBA strain. These results suggest that, at the 750mg/L dose and for the CBA strain, the stomach and intestinal mucosa of the adult is effective in protecting against the absorption of Al or that any Al which is absorbed is cleared by the kidneys. For this reason the dose of Al was increased to 1000 and 1250mg/L, and the treatment period extended to include all of gestation. Increasing the dose to 1000mg/L caused a reduction in birth weight but this effect on body weight did not persist for any experimental group. However, at the 1250mg/L dose, CBA pups fostered to treated mothers weighed more during the preweaning period than those reared by control females.

Within the C57 strain, pup weights at birth were influenced by oral exposure to Al at a dose of 1000mg/L only; treated pups weighing less than controls. Following exposure at the lowest dose (750mg/L), the body weight of C57 pups fostered to treated mothers up to weaning was greater than those reared by controls. This obesity continued into adulthood for male subjects and was only apparent in females after 13 weeks of age. Schroeder and Mitchener (1975) found a slight elevation in the body weight of male Long-Evans rats after exposure to 5ppm aluminium phosphate for one year. Similarly, Golub, Han, Keen and Gerschwin (1992) reported a transient increase in growth of female outbred Swiss-Webster mice between 8-12 weeks following 90 days of exposure to a diet containing 1000 ug of AlLact from 4 weeks of age. These findings emphasise the importance of testing subjects over their lifespan to reveal any latent effects.

At the highest dose (1250mg/L) the opposite effect on C57 pup body weight was true; pups fostered to treated mothers weighed

less than controls. This paradoxical effect of dosage on body weight is in agreement with that previously reported by Yokel (1984, 1985), following exposure of New Zealand white rabbits to AlLact during gestation (Yokel, 1985) and lactation (Yokel, 1984). Exposure of rabbit offspring to low levels of Al facilitated weight gain, whilst exposure at higher levels resulted in a decrease in body weight of treated subjects compared to controls. From the maternal behaviour observations at the 1250mg/L dose, C57 pups reared by treated mothers received less maternal care during the first postnatal week compared to pups suckling control mothers. This finding may explain the differences in body weight of pups reared by treated mothers. Al may also have affected the quality and quantity of milk output.

In conjunction with the weight data at the 1250mg/L dose, the maternal treatment effect influenced CBA pup performance in *forelimb grasping*; by Pd12 a greater proportion of pups reared by treated mothers acquired an adult response. As there was some correlation of body weight with Fox test score, these differences may have resulted from the increased weight of CBA pups reared by treated mothers rather than a direct effect of exposure to Al. This effect was also true for C57 pups treated with Al at a dose of 750mg/L. However, at the 1250mg/L dose the trend was towards C57 pups reared by treated mothers exhibiting deficits in performance. Again, this difference may be related to the lower body weight of these pups. Deficits in performance in neuromotor tests following oral exposure to Al have been more commonly reported (Tsujii and Hoshishima 1979; Bernuzzi, *et al.*, 1986, 1989; Donald, *et al.*, 1989; Muller, *et al.*, 1990). However, at the 1250mg/L dose

the trend was towards C57 pups reared by treated mothers exhibiting deficits in performance. Again, this difference may be related to the lower body weight of these pups.

Exposure to Al via the oral route did not produce as dramatic an effect on CBA ultrasonic calling as was found following i.p. exposure. There was nevertheless a trend towards treated CBA pups calling less than Cc pups at both doses tested (1000 and 1250mg/L). Further, at the higher dose the calling output of treated pups peaked later than controls.

The strain differences in ultrasonic calling are clearly evident from graphs 6.2 and 6.3, CBA pups exhibited at least a 10-fold increase in calling compared to C57 pups. Thus, the C57 strain may not be as useful a strain for the recording of ultrasonic calling as part of a test battery because of its innate low level of calling. This produces a floor effect: that is as the baseline rate of calling is so low, any differences induced by exposure to a toxicant are less likely to be clearly seen.

The results from the maze test are inconclusive. In the case of CBA mice it suggests that exposure to the doses employed (750 and 1000mg/L) resulted in only slight alterations to cognitive function; treated males required more days to reach criterion. Within the C57 strain, there was a trend towards treated males actually performing better than controls. The challenge with scopolamine produced the predicted detrimental effects on performance in the maze in control and treated subjects. However, the effect of scopolamine on treated C57 mice seemed to wane by the third exposure. This suggests that these animals were less sensitive to its effects or became tolerant to them, and implies that neurotransmitter

systems other than the cholinergic may be more important in mediating performance in the maze after exposure to Al in this strain. For example, as suggested in chapter 3, exposure to Al early in development may have initiated compensatory mechanisms which were already activated by 10 weeks of age.

Improvements in maze performance following drug exposure have been reported previously. Benton, Dalrymple-Alford, Brain and Grimm (1985) found that prenatal exposure of TO mice to diazepam (2.5mg/kg body weight) increased the number of correct responses compared to controls. These authors accounted for this difference in terms of diazepam altering some other aspect of behaviour which facilitated maze performance, rather than a direct effect on learning and memory.

From a methodological point of view, reducing the number of arms in the maze to six did not improve overall performance. Reinstein, DeBoissiere, Robinson and Wurtman (1983) found that C57BL/6J mice did not perform better than chance levels in an 8-arm radial maze. This is in contrast to a study reported by Ammassari-Teule and Caprioli (1985), which showed that DBA/2 and C57BL/6J inbred strains of mice learnt a 6-arm radial maze and furthermore, C57BL/6J mice performed better than DBA/2. Although the latter study also used food as the reinforcer, in future experiments with mice, it may be more profitable to use a liquid reinforcer as the motivator than food deprivation, as has been successfully employed by other workers (Pick and Yanai, 1983, 1985; Laviola, Pick, Yanai and Alleva, in press). In addition, a pilot study has shown that C57 males exposed to Al by i.p. injection

performed less well in an 8-arm radial maze when the width and height of the arms were reduced.

Differences in neurochemical measures depending on the sex (Yanai, 1979; Luine and McEwen, 1983; Luine, Renner, Heady and Jones, 1986) and strain (Gilad and Gilad, 1981; Gilad, Rabey, Tizabi and Gilad, 1987; Waller, Ingram, Reynolds and London, 1983) of a subject have been reported previously and for other neurotransmitter systems (Tunnicliff, Wimer and Wimer, 1973). Following exposure to oral Al at a dose of 1000mg/L, females tended towards an increase in ChAT activity compared to males at 23 weeks of age, especially within the CBA strain. Strain-dependent differences were not as obvious as the differences between the sexes. The many inconsistencies and anomalous results make only general observations possible. These inconsistencies may have resulted from individual variation in ChAT levels. Other workers have found considerable differences in ChAT levels between individuals of the same sex and strain (Sedowofia, pers. comm.).

The trend within CBA males was towards a decrease in ChAT levels following Al exposure whether the exposure occurred *in utero* or during the postnatal period following fostering to a treated mother. This trend was also present for CBA females in the **Ct** and **Tc** groups, but in contrast **Tt** females tended towards an increase in ChAT levels.

The seemingly consistent trend found in the CBA strain, of exposure to Al reducing the levels of ChAT activity, is not as obviously apparent within the C57 strain. C57 males seem to be the most variable group, with the effect of Al highly dependent on brain region. In contrast, the trend for C57 females is similar to that found

for the CBA strain apart from some inconsistent results within the **Ct** group. Thus, the ChAT data demonstrates that oral Al can penetrate the immature blood-brain barrier and reach the central nervous system of the developing foetus. Indeed, even at the 750mg/L dose regionally specific effects on ChAT activity were found (Sedowofia, pers. comm.). In one of the few studies which has considered Al's effects on neurochemistry, Marquis (1982) reported a reduction in AChE activity in Sprague-Dawley rats who were nursed by mothers exposed to a diet containing 0.12% Al chlorohydrate.

Interestingly, at 23 weeks of age, CBA females and C57 males within the **Tt** group exhibited increased levels of ChAT activity. One might speculate that Al's mechanism of action may be similar to that of excitotoxins: that is Al may have an excitatory effect causing overstimulation of neurons which initially results in an increase in, for example, neurotransmitter release, until a critical point is reached after which the neurons die. This stimulation may be a direct effect of Al on neurons or indirectly via the production of excitatory amino acids. If ChAT activity had been assessed at a later age, levels within these susceptible regions may have been lower compared to controls as neuronal excitation will have given way to neuronal death.

Presumably the penetrance of Al and the susceptibility to it, may vary between brain regions which explains the lack of uniform response and why certain brain regions are more susceptible than others. The biphasic response to Al e.g. in the body weight of C57 pups, may have resulted from a stimulatory effect of exposure to low levels whilst exposure to higher levels had a depressant effect. There is some evidence to support this (Atterwill, 1989). *In vitro* treatment

of whole-brain reaggregate cultures with $AlCl_3$ at a concentration of 0.01mM and 0.1mM, resulted in increased ChAT activity up to 48 hours of exposure but thereafter activity was reduced compared to control values (Atterwill, 1989). To lend further support to this hypothesis it would be necessary to measure the Al levels in different brain regions and to correlate these with ChAT activity.

In some instances e.g. within the midbrain of CBA males, postnatal exposure via the dam's milk seems to have had a greater effect on ChAT activity levels than prenatal exposure. Many systems are still developing during the postnatal period and this may enhance their susceptibility to Al's action.

In general, it would seem that less risk is attached to exposure to Al via the oral route than from injection. The reduced effect of oral Al, especially within the CBA strain, was somewhat surprising considering the larger dose of Al consumed over a longer treatment period compared to i.p. exposure. However, this reduced effect reinforces the argument that the increased detrimental effects of injected Al resulted from the continued leakage of Al from tissue surrounding the injection site. Al administered by this route would pass directly into the bloodstream, thus increasing its bioavailability. On the other hand, oral Al passes into the stomach where its absorption may be prevented or slowed down, so that the foetus is exposed to much lower doses.

Cranmer, Wilkins, Cannon and Smith (1986) have reported the Al content of the placenta and the foetus of BALB/c mice, exposed to oral $AlCl_3$ at a dose of 200mg/kg/day during days 7-16 of gestation, to be twice that of controls. Thus, although the absorption of Al may be restricted by the gastrointestinal tract, a

proportion of the oral Al must be adsorbed, contrary to previous assumptions outlined in chapter 1, to produce the effects described here. The placenta may be effective in eliminating low levels of absorbed Al but its ability to prevent Al from entering the foetus is reduced when levels of Al are higher.

CHAPTER 7

7.1 GENERAL DISCUSSION

The vulnerability of the developing foetus to exposure of the mother during pregnancy to certain drugs and chemicals present within the environment, has led to the recognition of a wide variety of behavioural teratogens which can affect intrauterine and postnatal development. The results of the experiments presented in this thesis suggest that Al could be added to this list. This is of particular concern with respect to the increasing levels of Al which are entering the environment from industrial sources (Wide, 1984).

The main limitation to this study has been the lack of measurements of levels of Al within various maternal and foetal body tissues, which would have provided definitive evidence that Al was absorbed and accumulated within these tissues. Data relating to the Al content of milk, foetal tissues and within different brain regions, would have enabled correlations to be made between alterations in behaviour and neurochemistry with the effective levels of Al present. Attempts were made over the course of this work to take such measurements, but they resulted in anomalous results probably due to the contamination of control material, either from airborne Al or from the presence of Al in the dissecting instruments.

The underlying diminution of maternal weight gain during gestation following the treatment of CBA females with injected Al, is an undesirable confounding variable. It could be argued that this effect of Al on the mothers may have contributed to the changes in physical and behavioural development of their offspring as a result of undernutrition rather than a direct effect of exposure to Al. Certainly, the differences in maternal weight gain may account for

the body weight differences between control and treated pups at birth. However, as dams were not found to differ in their food and water intake or in body weight during lactation, the contribution of differences in maternal weight to the physical and behavioural differences between the experimental groups up to weaning, seems an inadequate and unlikely explanation.

The most obvious effect of prenatal exposure to Al has been the influence of the treatment received by the foster mother on aspects of offspring development. This suggests that postnatal exposure to Al is of more consequence than prenatal. The extent to which Al accumulates and persists in body tissues is not known, but it seems certain that some Al is available to distribute into the dam's milk, resulting in continued exposure of the neonate over time. In the human and rodent, the maturation of many neurochemical systems continues postnatally. For example, granule cells within the cerebellum and hippocampus proliferate during the postnatal period (Ruppert, 1987). This may explain why postnatal exposure to Al, via the dam, continues to have effects on offspring development. Further, as gestational exposure to Al caused only slight changes in certain components of maternal behaviour, the contribution of alterations in the ability of the mother to care for her fostered young to the maternal effect is ruled out.

Whatever the mechanism of action, the presence of a maternal effect highlights further how essential it is to incorporate a cross-fostering procedure in behavioural teratology studies if any safe deductions about mechanisms are to be made.

In utero exposure to Al via i.p. injections reduced the number of ultrasonic calls produced and delayed the timing of peak

calling in CBA treated pups compared to controls (see chapter 4). A similar trend towards a diminished calling output was found following oral exposure but the results were not as striking. Pups who received Al *in utero* but were reared by control mothers (Tc), were one of the heaviest groups but called at a rate lower than controls. This suggests that the effect of Al was specific to disruption of the vocalisation system. The exact neural mechanisms underlying ultrasonic calling are not known and the involvement of several systems has been implicated. The demonstration of a reduction in ChAT activity following prenatal exposure to Al, leads to the tentative suggestion that the cholinergic system may play a role in the production of these calls (Rankin and Manning, 1993). Obviously further work is required to investigate the consequences of a lowered calling rate on the mother-infant interaction and to understand Al's mechanism of action. However, as suggested in chapter 4, recording of ultrasonic calling should certainly be included in any test battery aimed at assessing effects of possible behavioural teratogens on the developing animal.

CBA pups exposed to injected Al exhibited transient delays in several tests of neuromotor development, specifically in *forelimb*, *pole grasping* and *screen climbing*. Following exposure via the oral route, Al-treated CBA pups performed better in some of the Fox tests than controls. Information concerning the effects of injected Al on the neurobehavioural development of C57 pups is lacking.

As has been reported by other workers, there was a paradoxical effect of dosage on body weight following exposure by the oral route within the C57 strain. C57 pups exposed to the lowest dose of oral Al (750mg/L) weighed more than controls and also

acquired a mature response earlier than controls in some of the Fox tests. Conversely, when exposed to a dose of 1250mg/L oral Al, treated C57 pups had the lowest body weight which was accompanied by delays in the maturation of some of the neurobehavioural tests. Thus, performance in the Fox tests is related to body weight.

Although the results from the maze experiments were inconclusive, they do suggest that prenatal exposure to Al disrupts some aspects of maze learning. Mice certainly seem to be less efficient in acquiring this type of learning task compared to rats. Mice may be more sensitive to the physical characteristics of the maze such as the dimension of the alleys or the light intensity. In a pilot experiment which involved an 8-arm radial maze with reduced dimensions, i.p. Al-treated C57 males required significantly more trials to complete the task compared to controls. Further, the task may have proved to be too difficult for all the mice so that treatment effects were not obvious. The results of the maze experiments, the adult activity tests and adult body weights, suggest that *in utero* exposure to Al does not only affect aspects of neonatal development but that certain effects persist into adult life.

Measurement of the levels of ChAT activity showed that changes induced by exposure to Al depended upon brain region, age, strain and sex. Exposure via i.p. injection resulted in consistent reductions in activity within the cerebral cortex and hippocampus in treated CBA brains compared to controls at all ages tested. It is of interest to note that the brain areas which undergo the most consistent changes in Alzheimer's disease are the cerebral cortex, hippocampus and nucleus basalis of Meynert (Mann, 1988). There

is no information regarding the effect of injected Al on ChAT levels within different regions of C57 brains. This resulted from the insufficient number of plugged C57 females, a problem which has been encountered by other workers using this inbred strain. When exposure occurred via the oral route, there was a tendency towards a decrease in ChAT activity within the two strains, although deviations from this general trend were clearly apparent. Similar reductions in cholinergic system enzymes have been reported following exposure to other heavy metals. Sobotka, Brodie and Cook (1975) reported decreases in AChE and butyrylcholinesterase enzyme activities in the telencephalon and brainstem following neonatal exposure of rats to lead acetate (9, 27, 81mg/kg). Exposure of Sprague-Dawley rats to methylmercury at a dose of 2 or 6mg/kg during gestation days 6-9, caused a reduction in AChE activity in the cerebral cortex, brainstem and cerebellum when assayed at Pd12 (Wootten, Brown, Callahan, Vetrano, Wadman, Melia, Mulligan and Schatz, 1985).

The marked differences in the levels of ChAT within control and treated brains implies the involvement of the cholinergic system in the effects described. However, it is unlikely that the cholinergic system is the only system affected by Al and it is more plausible that a number of neurochemical systems were altered. However, with the association of Al and alterations to the cholinergic system within the Alzheimer's diseased brain, a marker for this system was the most obvious one to include in initial studies.

The degree of loss of ChAT activity and the extent of physical and behavioural change are incongruous. In chapter 3 this inconsistent level of change was explained in terms of neuronal plasticity and compensatory mechanisms. It certainly seems from

previous studies (Yates, Gordon and Wilson, 1976; Bayer, 1989), that a high degree of neuronal loss can be overcome before behaviour is disrupted. This may be especially true in the case of immature animals, the nervous system of which is undergoing continuous change.

Here, as in almost every study which has explored strain differences, susceptibility to a number of drugs has been shown to depend on the subject's genotype. There have been some clear genotypic differences in the response to exposure to Al by mice from the CBA/T6 and C57BL/6J inbred strains. For example, the body weight of CBA pups was unaffected by oral exposure to Al at a dose of 750mg/L. On the other hand, similar exposure of C57 pups resulted in pups fostered to treated mothers weighing more than pups reared by control females.

The exact means by which Al exerts its developmental toxicity is not known but several possible mechanisms may be put forward. For example, the mode of action may be similar to that of cadmium, which interferes with the placental transport of certain vitamins, particularly vitamin B_{12} (Ullberg, Dencker and Danielsson, 1982), although there may be other more general effects on nutrient transport at the placental level which may be affected by Al. This would result in difficulties for the exchange mechanisms between the mother and the foetus. Such deficits in the transport of essential elements to the developing foetus, would certainly explain the reduced body weight of treated pups at birth. If these prenatal deficits are transient, then pups given adequate maternal care during lactation may overcome such deficits. This would account for pups

in the **Tc** group having comparable body weights to that of control pups reared by control mothers (**Cc**).

The retarding effect of Al on the growth of **Ct** and **Tt** pups may have resulted from differences in the quality and quantity of milk produced by treated mothers. This effect would be enhanced if difficulties in intestinal absorption and metabolism continued beyond birth. Indeed, Al is known to inhibit gastrointestinal tract mobility (Hava and Hurwitz, 1973) which may cause undernutrition.

The question still remains of how Al gains entry to the CNS to exert its toxicity. The absorption of Al is dependent on the ligand to which it is bound and its penetration of the body's barriers is facilitated by its complexing with citrate (Slanina, Frech, Ekström, Lööf, Slorach, and Cedergren, 1986; Martin, 1986) and maltolate (Kruck and McLachlan, 1989). Further, Al binds to albumin (Martin, 1986) and transferrin (Trapp, 1983; Martin, Savory, Brown, Bertholf and Wills, 1987) and can be transported within the body in this way. Banks and Kastin (1983) have demonstrated that Al alters the permeability of the rat blood-brain barrier, which allows the entry of substances whose access is normally restricted. More specifically, Banks, Kastin and Fasold (1988) have shown that Al inhibits saturable transport systems in the blood-brain barrier. Thus, neurons may be affected directly by exposure to Al and killed by a process of overstimulation, as suggested in chapter 6, or degeneration may be incurred by exposure to other substances present in the blood, the entry of which is facilitated by Al.

In summary, the involvement of Al in the aetiology of a number of human pathological conditions in more recent years, has changed opinions on its previously established status as a nontoxic,

nonabsorbable, harmless element. To date, little attention has been given to Al's potential as a behavioural teratogen. It is clear that we still lack information on a number of behavioural systems following gestational exposure to Al, which severely limits a more comprehensive evaluation of Al's behavioural teratogenicity. However, the results presented herein, and those of other investigators, imply that prenatal exposure to Al causes alterations to growth, ultrasonic calling, to the development of certain sensory-motor skills, and suggests changes to maternal behaviour and learning and in the levels of ChAT activity in certain brain regions. Moreover, such exposure may result in behavioural and neurochemical deficits which persist into adulthood.

Finally, Al may be poorly absorbed from the gastrointestinal tract under normal conditions and at low levels, but in the developing animal such protective biological barriers are immature which may render them particularly susceptible to any toxic effects. Further work is certainly necessary for a better understanding of the influence of Al on foetal development.

7.2 DIRECTION OF FUTURE WORK

As is often the case with scientific research, the work presented here has raised as many questions as it has answered. Al is present in the environment in a number of different chemical forms. The extent of the solubility of Al in the body is dependent on the Al salt to which exposure results (LeBlondel and Allain, 1980; Martin, 1986), thus the need for evaluation of inter- and intraspecies differences in the bioavailability of Al and its salts is great.

The response to a drug is highly dependent on the species tested. It may be informative to expose animals from a different species, to Al to assess the generality of Al's effects. For example, Chapman and Cutler (1988) found that, for certain measures, the gerbil (*Meriones unguiculatus*) was affected to a relatively greater extent by prenatal exposure to phenobarbitone (60mg/kg) in their mother's drinking water, than the mouse.

Further investigations are needed to examine the effects of gestational exposure to Al on the mother-infant interaction. For example, does *in utero* exposure to Al make a treated pup intrinsically different from a control pup as a stimulus object, making it less effective in eliciting maternal behaviour? To do this, one could present unfamiliar control and Al-treated pups to control and treated mothers, and measure the ultrasonic output of these pups and simultaneously record maternal behaviour and retrieval time, in a similar way to that employed by Ness and Franchina (1990) and Laviola, Rankin, Petruzzi and Alleva (subm.). Further, pups could be exposed to a different sensory stimulus e.g. olfaction, and the number of ultrasounds recorded. This would provide information on the specific effect of Al on the pup vocalisation system and eliminate any contribution from effects on the processes underlying thermoregulation.

This study has not included any effect of prenatal exposure to Al on social interactions. The main reasons for this concerns ethical considerations and practical problems involved in such experiments. A further study could include a similar procedure to that used by Chapman and Cutler (1984, 1988) involving the

recording of a range of social behaviours during single sex encounters between unfamiliar animals.

Thus there are fundamental problems concerned with the understanding of behavioural development, for example genotype/environmental interactions, which the ethopharmacological approach may help to illuminate. But also, from a practical point of view, we must continue to test possible teratogens to identify behavioural anomalies early enough to prevent further human tragedies from occurring.

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APPENDICES

APPENDIX A

LONG-TERM EFFECTS OF ALUMINIUM ON THE FETAL MOUSE BRAIN

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Summary

Potentially noxious substances may act as fetal teratogens at levels far lower than those required to produce detectable effects in adults, and behavioural teratogenicity may occur at levels lower than those which produce morphological teratogenesis. Aluminium (Al) is a potential neurotoxin in adults. Since pregnant women may be exposed to untoward levels of Al compounds under certain conditions, we have examined the long-term effects of treating the pregnant mouse with intraperitoneal or oral aluminium sulphate on brain biochemistry and behaviour of the offspring. The cholinergic system, as evaluated by the activity of choline acetyltransferase (ChAT), was affected differentially in different regions of the brain, and still showed significant effects in the adult. Differences between the intraperitoneal and oral series in the magnitude of effect seen in the regions of the brain probably reflect differences in the effective level of exposure. Growth rate and psychomotor maturation in the pre-weaning mouse were affected in the intraperitoneal series only, showing a marked post-natal maternal effect.

The presence of aluminium (Al) compounds in the brains of sufferers of senile dementia of the Alzheimer type (SDAT) has led to the suggestion that Al accumulation in the brain leads to degenerative processes(1). Although Al is the third most abundant element, most of it exists in insoluble forms. The limited passage of Al salts across the gut, the efficient excretion by the kidneys, and the blood-brain barrier diminish the access of soluble Al compounds to the brain(2). However acute or chronic administration of Al compounds to experimental animals by various routes, including the oral(3-5), has established the neurotoxicity of soluble aluminium compounds, as do human conditions such as dialysis encephalopathy(6,7). Many of the neurological diseases in which Al has been implicated are those in which there is damage to the cholinergic system(8), and there is evidence of cholinergic damage in conditions where aluminium has been introduced experimentally into the brain(5,9,10). Al compounds are more readily taken up at acid pH or in organified form. For example Al uptake is increased by citrate(11,12) or by complexing with sugars(13,14). Such organification may permit solubility in cell membranes, thus affecting permeability and the blood-brain barrier(15). Aluminium may bind to receptor

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tes for several neurotransmitters, to membrane phospholipids, or to lmodulin, thus affecting calcium homeostasis(16). Pregnant women may be posed to aluminium compounds in several industries(8), or from ironmental pollution in the vicinity of aluminium smelters, or following cidents (several of which have been reported in the U.K.) with aluminium lphate added to water supplies. The fetus is in general susceptible to lower concentrations of noxious substances than adult tissues(17), and se substances may act as behavioural teratogens, producing long term or manent deficits in brain function, often at even lower levels than those quired to produce morphological teratogenesis(18). We have used mice ich, like humans, have a haemochorial placenta, to study the effects of enatal exposure to aluminium sulphate on brain biochemistry and behavioural velopment, and report here on long-term effects on growth, behaviour, and cholinergic system. The effect on the cholinergic system, as assessed by asuring the activity of choline acetyltransferase (ChAT) was chosen because the known effects of Al on this neurotransmitter system(9,10).

Materials and methods

Administration of aluminium Aluminium was administered to pregnant mice by two routes. One group of mice was injected intraperitoneally p) with 200mg/kg aluminium sulphate ($Al_2(SO_4)_3.18H_2O$, pH 2.5-2.7) or line acidified to the same pH as the aluminium, from days 10-13 of station inclusive. The second group was given aluminium sulphate (750mg/l., 4.1-4.3) as their sole drinking water from days 10-17 of gestation clusive. Control mice in this group were given water acidified to the same as the aluminium sulphate.

Determination of choline acetyltransferase activity Control and uminium exposed mice were killed at ages ranging from 3-44 weeks. The ains were dissected into different regions including cerebral cortex, rebellum, hippocampus, midbrain, hypothalamus, nucleus magnocellularis, riatum and the brainstem. Pooled samples from 2-4 mice (not separated by x), were stored at -70°C until used for the determination of ChAT activity the method of Glover and Green (19). Briefly, samples were homogenised on in saline containing 1% butan-1-ol, and assayed with an assay mixture taining ^{14}C acetylCoA at 37°C for 20 mins. ^{14}C acetylcholine formed was tracted into octan-2-one containing potassium mercuric iodide, and aliquots unted in a scintillation counter. Each group of age-matched control and ated brains was dissected and analysed together. However, the oral series re begun a year after the intraperitoneal series, and dissections were done a different operator. Thus while each control and treated group is wholly mparable, the controls for the intraperitoneal and oral series are not mparable.

Behavioural and developmental studies On the day of birth (post-natal y zero, Pd0) litter sizes were recorded. The following day pups were ighed, sexed, marked by toe clipping and cross-fostered using a total lit-litter cross-foster design, and weighed on every third day from Pd3 ll weaning (see ref. 20). Thereafter, body weights were recorded weekly. le and female weights were analysed separately from weaning onwards. Tests sensori-motor coordination and development were also performed every ird day using a modified version of the Fox battery of tests (21). Subjects re tested for slow righting, cliff aversion, grasping with forelimbs, pole asping, climbing on a wire mesh and eye opening. All other behavioural

tests were performed as previously described (20), except for the test of learning ability. In these experiments, 10 week old male mice were tested in an 8-arm radial maze for 12 days as described by Pick and Yanai (22). Adult activity tests on three successive days were carried out at 22 weeks (see 20).

The following four experimental groups of mice were established and tested:- (1) pups from control litters fostered to control mothers (Cc), (2) pups from control litters fostered to Al treated mothers (Ct), (3) pups from Al treated litters fostered to control mothers (Tc), and (4) pups from Al treated litters fostered to Al treated mothers (Tt). All the behavioural studies were carried out by the same observer who was blind to the allocation of treatment groups. The sample size for each group, including males and females, in the i.p series was as follows:- Cc n=13, Ct n=14, Tt n=12, Tc n=11; and for the oral series Cc n=8, Ct n=9, Tt n=10 and Tc n=8.

Statistical analysis Pup weights at birth were analysed using Student's *t*-test. Analysis of Variance (ANOVA) was employed for the weights and activity scores thereafter (23). Adult activity data was analysed using the Kruskal-Wallis one-way ANOVA. The categorical data generated from the Fox tests was assessed by Chi-square tests on each day of observation. ANOVA followed by *t*-test was used to analyse ChAT activity data. Differences at $p<0.05$ were taken as significant.

Results

Levels of aluminium consumed or injected

With an average weight of 25g, pregnant mice injected intraperitoneally with aluminium sulphate received a daily dose of 0.4mg of elemental aluminium. The mean amount of fluid consumed daily by control mice in the orally exposed group was 6.0ml. This compares with a mean of 4.4 ml consumed by the treated mice, and is equivalent to a daily intake of 0.27mg of elemental aluminium. Over a four-day treatment period, i.p mice received a total of 1.64mg of aluminium, compared with 1.89mg consumed by orally exposed mice in seven days.

Neurochemistry

Our data indicate that pre-natal exposure to aluminium sulphate affects the cholinergic system, as judged by effects on choline acetyltransferase activity. Table 1 shows ChAT activity in different brain regions from mice whose mothers were administered aluminium sulphate by intraperitoneal injection. The data in table 2 show the activity in mice whose mothers were exposed orally to aluminium. Both tables show significant effects on the cholinergic system, and also that the effects are differential between brain regions. It is also clear from the data that, with the exception of the hypothalamus and striatum, the effects persist into adulthood.

Developmental and behavioural effects

There were no effects on the length of gestation, litter size or sex ratio of either Al treatment. The birth weights of exposed pups in the intraperitoneal series were about 5 per cent below controls ($df=1,47$ $t=2.63$ $p=0.011$) but this difference persisted only in the mice reared by treated mothers. Fig. 1 shows this clear maternal effect, and the increasing

divergence of pup weights reared by control or treated mothers from Pd3 up to weaning ($df=1,42$ $F=16.43$ $p=0.0002$). The maternal effect on body weight persisted into adulthood for female mice only ($df=1,18$ $F=8.42$ $p<0.01$). There were no significant effects on body weight of pups whose mothers were exposed to aluminium sulphate orally during gestation.

Pups exposed to aluminium via the i.p route were slower to attain adult scores on several of the Fox scale measures of reflex development(21): including slow righting, forelimb grasping (Fig. 2) and pole grasping (Fig. 3), all $p<0.05$. In addition, we have found a maternal effect on sensori-motor performance. Figs. 2 and 3 show that treated pups reared by treated mothers (Tt) are more severely affected than treated pups reared by control mothers (Tc) or control pups reared by treated mothers (Ct), but all these groups are affected as compared to controls reared by control mothers (Cc). The divergence between the two extreme groups, control pups reared by control mothers, and treated pups reared by treated mothers is the widest. The significance for pole grasping, for example, is $p<0.001$. The other tests were not found to be significant. Open field activity levels were largely unaffected up to weaning.

TABLE I

CHOLINE ACETYLTRANSFERASE ACTIVITY IN CBA MICE EXPOSED PRE-NATALLY TO ALUMINIUM SULPHATE

	3 weeks		17 weeks	
	CONTROL	TREATED	CONTROL	TREATED
CEREBRAL CORTEX	213.4± 9.3	210.9± 4.8	440.3± 2.6	328.9± 8.3*
CEREBELLUM	43.3± 0.7	50.7± 3.1	98.5± 2.7	64.9± 1.9*
HIPPOCAMPUS	744.4± 7.6	580.2± 8.2*	1015.6±31.0	917.9± 3.6
MIDBRAIN	N.D.	N.D.	793.3±13.1	856.6± 3.9*
HYPOTHALAMUS	N.D.	N.D.	665.7±19.9	573.9±26.7
STRIATUM	459.6± 9.7	251.5± 5.2*	2141.7±52.7	1871.4±43.9*

	34 weeks		44 weeks	
	CONTROL	TREATED	CONTROL	TREATED
CEREBRAL CORTEX	309.4±22.0	180.6± 1.4*	247.0± 5.3	215.4± 2.5*
CEREBELLUM	50.9± 1.1	46.5± 0.9*	73.2± 1.1	45.6± 1.0*
HIPPOCAMPUS	381.1±18.1	306.7± 1.4*	567.2± 6.0	545.5±10.0
MIDBRAIN	649.4± 7.8	552.1± 6.3*	505.7±15.0	439.8± 3.8*
HYPOTHALAMUS	530.9± 2.1	433.8± 6.3*	247.5± 2.4	342.2± 9.0*
STRIATUM	1047.7±24.3	995.0± 7.0	431.7± 8.4	728.2±16.0*

Choline acetyltransferase activity in mouse brain. Brains were dissected from offspring whose mothers were injected intraperitoneally with 200mg/kg aluminium sulphate or saline on gestation days 10-13. Enzyme activity expressed as picomoles/mg protein/minute. Each result represents the Mean ± SE of 4 determinations. Values *$p<0.05$; N.D.= Not Determined.

Measurements of activity at weaning and in adult life show persistent trends towards a maternal treatment effect. At 22 weeks, a five minute activity test was performed. There was no significant difference between groups on day 1 when they were introduced to the field. However, there were marginally significant differences between treatment groups in the total number of squares crossed on days 2 and 3, pups reared by treated mothers had lower activity levels.

TABLE II

ChAT ACTIVITY IN CBA MICE EXPOSED TO ALUMINIUM SULPHATE PRE-NATALLY (ORALLY ADMINISTERED TO MOTHERS FROM DAY 10-17 OF PREGNANCY)

	3.5 Weeks		16 Weeks	
	CONTROL	TREATED	CONTROL	TREATED
CEREBRAL CORTEX	197.3± 3.8	322.9± 2.5*	516.5±12.1	358.2± 5.0*
CEREBELLUM	105.5± 1.2	61.4± 4.1*	97.6± 2.3	87.3± 0.8*
HIPPOCAMPUS	568.9± 8.5	661.5±26.6*	678.7± 5.8	725.9±13.0*
MIDBRAIN	733.9±18.6	498.1± 9.1*	542.7± 6.1	562.8± 6.7*
HYPOTHALAMUS	338.4± 9.0	442.7± 2.7*	564.4± 8.7	478.8± 3.6*
NUCLEUS	1289.1± 5.3	1173.9±14.6*	1695.2±13.5	1415.1± 4.5*
STRIATUM	831.6± 7.7	751.2± 9.9*	1426.4± 5.4	868.3±10.5*
BRAINSTEM	870.7±12.8	890.9± 6.2	841.1± 7.1	977.6±45.9*

Choline acetyltransferase activity in mouse brain. Brain dissections and measurement of ChAT activity were as in table 1. Values *$p<0.05$ were significantly different from controls.

Tests at 10 weeks of age for learning ability and retention in an 8-arm radial maze could be made with small numbers only. 10 out of 13 control animals (77%) reached criterion [two consecutive errorless trials (22)] and 6 out of 11 (55%) of the Al-exposed group. The exposed mice which did reach criterion were slower to do so (a median of 7.5 days, compared to 6 days for controls), but this difference did not reach statistical significance.

Discussion

In both series the effects on ChAT levels following prenatal exposure to Al are differential between brain regions: a finding also reported for the neurotoxic effects in Al encephalopathy in adult rats (24). Table 2 shows that significant effects are found in ChAT activity following administration by the oral route, implying sufficient access of aluminium to the fetal brain for neurotoxic effects to be obtained.

It is clear from the data on ChAT that the effects of aluminium on the developing brain are not transient, but persist into adult life, although there are deviations from this trend in the striatum and hypothalamus in the oldest animals. The differences in the extent to which ChAT activity was affected in the different regions of the brain may be due to the levels of aluminium reaching each region, or to differences in the distribution of cholinergic neurons in the brain.

There were also differences in the effects on ChAT when the same regions of the brain are compared in the intraperitoneal and oral series.

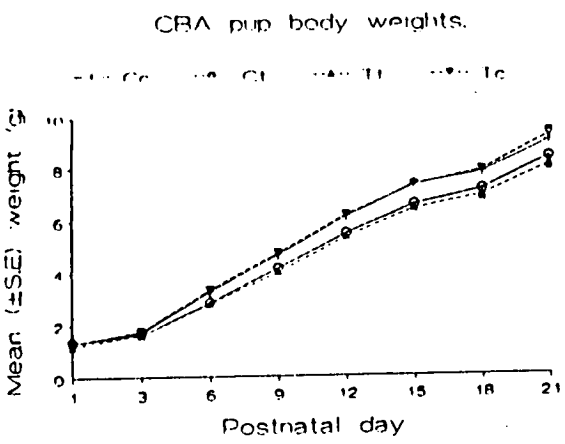
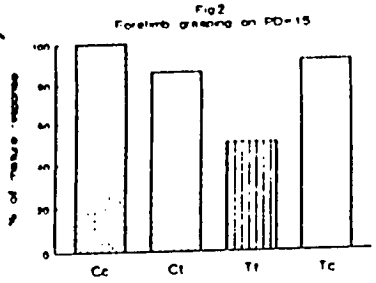
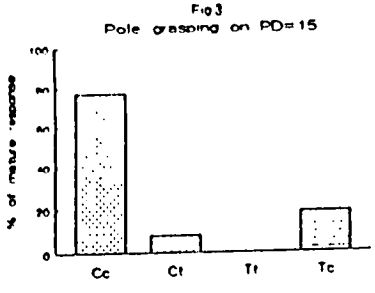


Fig. 1

Effects on the body weight from birth to weaning of mice pups whose mothers were exposed by intraperitoneal injections of aluminium sulphate (200mg/kg) on gestation days 10-13. Cc - Control pups reared by control mothers; Ct - Control pups reared by treated mothers; Tt - Treated pups reared by treated mothers; Tc - Treated pups reared by control mothers.





This may reflect a dose effect. It has been shown that aluminium levels in the placentas of pregnant female mice injected intraperitoneally were 10 times higher than in controls, while they were only three times higher than controls in orally treated females(25). Also a rise in performance in a classically conditioned reflex test(26) has been reported in rabbits following low levels of Al administration with a fall following high levels. Effects of prenatal aluminium on post-natal growth have also been reported by several other workers (27-31), and other investigators have reported similar results to ours, in that Al treatment *in utero* affects the development of certain but not all sensori-motor skills (32,33). Treatment of infant mice similarly affects motor skills (27), and regular clinical administration of Al to human infants is neurotoxic (34).

The increasing divergence between the pup weights of offspring reared by control or treated mothers up to weaning, may be due to retention of aluminium by the mother and its release into the milk, to effects on milk output(26), or to effects on maternal behaviour(35), but the neurochemical sequelae would imply that the pups received Al in the milk. Although Yokel(26) found relatively low levels of Al in the milk of treated dams, it may be in organified complexes and thus readily absorbed by the neonate.

Although some behavioural measures show effects, others do not, and overall the behavioural effects do not seem commensurate with the degree of ChAT deficit. Such disparities can also occur in SDAT brains (36,37). It must be pointed out that responses to depressed levels of ChAT may be highly selective amongst different types of memory (38) so that our battery of tests (20) and the effects on mice do not permit us to predict any particular loss of mental functions in man. However, Adams (39) has pointed to the similarity of behavioural responses in humans and rodents after exposure to several different types of behavioural teratogens, suggesting that mice may provide a very suitable model for behavioural teratogenicity in humans. It may also be that reduced ChAT activity increases the liability of the system to other forms of stress, a possibility which requires investigation.

Finally we have clear evidence for genetic differences in response to teratogens,(17,20,40) and for sensitivity to aluminium. Pregnant C57 mice, given the same intraperitoneal dose levels as were used for the CBA genotype and reported here, failed to continue to term.

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APPENDIX B

Main analysis of variance (ANOVA) tables and medain (+lower and upper interquartile ranges). The tail probability value for the repeated measure interactions is the Greenhouse Geisser correction.

ANOVA table of maternal body weight of CBA females injected i.p. with aluminium sulphate (200mg/kg, Gd10-13).

SOURCE	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARES	F VALUE	TAIL PROB.
MEAN	23999.01788	1	23999.01788	3522.02	0.0000
TREAT	34.40706	1	34.40706	5.05	0.0461
ERROR	74.95397	11	6.81400		
DAY	273.60113	2	136.80057	50.13	0.0000
DT	10.40009	2	5.20004	1.91	0.1926
ERROR	60.03888	22	2.72904		

ANOVA table of CBA pup body weight following prenatal exposure to aluminium sulphate (200mg/kg, Gd10-13).

SOURCE	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARES	F VALUE	TAIL PROB.
MEAN	6566.47828	1	6566.47828	4350.63	0.0000
TREAT	0.23429	1	0.23429	0.16	0.6956
FMAT	23.05899	1	23.05899	15.28	0.0003
SEX	0.15242	1	0.15242	0.10	0.7522
TF	0.60917	1	0.60917	0.40	0.5287
TS	1.81962	1	1.81962	1.21	0.2785
FS	1.52212	1	1.52212	1.01	0.3210
TFS	0.01814	1	0.01814	0.01	0.9132
ERROR	63.39136	42	1.50932		
DAY	1797.97353	6	299.66226	3471.31	0.0000
DT	0.11414	6	0.01902	0.22	0.7683
DF	7.55227	6	1.25871	14.58	0.0000
DS	0.12234	6	0.02039	0.24	0.7556
DTF	0.10122	6	0.01687	0.20	0.7889
DTS	0.44752	6	0.07459	0.86	0.4106
DFS	0.36911	6	0.06152	0.71	0.4730
DTFS	0.05028	6	0.00838	0.10	0.8794
ERROR	21.75401	252	0.08633		

ANOVA table of CBA adult female body weights.

SOURCE	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARES	F VALUE	TAIL PROB.
MEAN	116216.53540	1	116216.53540	6900.57	0.0000
TREAT	26.34204	1	26.34204	1.56	0.2271
FMAT	160.27994	1	160.27994	9.52	0.0064
TF	6.19717	1	6.19717	0.37	0.5517
ERROR	303.14869	18	16.84159		
WEEK	2524.13494	12	210.34458	348.62	0.0000

WT	11.89850	12	0.99154	1.64	0.1431
WF	13.13340	12	1.09445	1.81	0.1040
WTF	3.06930	12	0.25577	0.42	0.8598
ERROR	130.32551	216	0.60336		

ANOVA table of CBA pup *activity* during postnatal days 9-18.

SOURCE	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARES	F VALUE	TAIL PROB.
MEAN	137954.05044	1	137954.05044	178.81	0.0000
TREAT	68.56514	1	68.56514	0.09	0.7671
FMAT	3080.07171	1	3080.07171	3.99	0.0522
SEX	167.26682	1	167.26683	0.22	0.6439
TF	350.43200	1	350.43200	0.45	0.5040
TS	1573.69329	1	1573.69329	2.04	0.1606
FS	119.62528	1	119.62528	0.16	0.6957
TFS	1113.73557	1	1113.73557	1.44	0.2363
ERROR	32403.14048	42	771.50334		
DAY	83477.87999	3	27825.96000	93.94	0.0
DT	403.79421	3	134.59807	0.45	0.6333
DF	601.97146	3	200.65715	0.68	0.5085
DS	904.35513	3	301.45171	1.02	0.3649
DTF	1249.89793	3	416.63264	1.41	0.2508
DTS	1036.15660	3	345.38553	1.17	0.3161
DFS	77.80410	3	25.93470	0.09	0.9137
DTFS	4070.33841	3	1356.77947	4.58	0.0134
ERROR	37321.01190	126	296.19851		

ANOVA table of the time spent *crawling* in a three minute openfield test during postnatal days 6-18.

SOURCE	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARES	F VALUE	TAIL PROB.
MEAN	735330.16158	1	735330.16158	433.45	0.0000
TREAT	224.60527	1	224.60527	0.13	0.7178
FMAT	4306.83157	1	4306.83157	2.54	0.1186
SEX	558.28490	1	558.28490	0.33	0.5693
TF	3416.66725	1	3416.66725	2.01	0.1632
TS	2643.54542	1	2643.54542	1.56	0.2188
FS	1228.83491	1	1228.83491	0.72	0.3995
TFS	93.81313	1	93.81313	0.06	0.8152
ERROR	71250.81096	42	1696.44788		
DAY	192756.22562	4	48189.05641	75.87	0.0000
DT	3654.58901	4	913.64725	1.44	0.2288
DF	5177.82837	4	1294.45709	2.04	0.1003
DS	1917.35168	4	492.83792	0.78	0.5282
DTF	4954.33415	4	1238.58354	1.95	0.1135
DTS	9470.04186	4	2367.51047	3.73	0.0088
DFS	2537.51467	4	634.37867	1.00	0.4041
DTFS	7092.89915	4	1773.22479	2.79	0.0342
ERROR	106704.77259	168	635.14746		

REARING

SOURCE	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARES	F VALUE	TAIL PROB.
MEAN	1434.77124	1	1434.77124	67.19	0.0000
TREAT	3.45997	1	3.45997	0.16	0.6893
FMAT	63.93616	1	63.93616	2.99	0.0909
SEX	6.43382	1	6.43382	0.30	0.5860
TF	8.97292	1	8.97292	0.42	0.5204
TS	16.47059	1	16.47059	0.77	0.3848
FS	5.37815	1	5.37815	0.25	0.6184
TFS	3.28256	1	3.28256	0.15	0.6970
ERROR	896.84127	42	21.35336		
DAY	462.04077	2	231.02038	21.29	0.0
DT	12.65106	2	6.32553	0.58	0.5261
DF	27.51591	2	13.75795	1.27	0.2828
DS	4.43666	2	2.21833	0.20	0.7687
DTF	7.60659	2	3.80330	0.35	0.6602
DTS	14.56166	2	7.28083	0.67	0.4843
DFS	14.03995	2	7.01998	0.65	0.4953
DTFS	106.78260	2	53.39130	4.92	0.0151
ERROR	911.36825	84	10.84962		

STILL

SOURCE	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARES	F VALUE	TAIL PROB.
MEAN	2748.23619	1	2748.23619	506.17	0.0000
TREAT	3.67148	1	3.67148	0.68	0.4155
FMAT	23.92506	1	23.92506	4.41	0.0419
SEX	0.02471	1	0.02471	0.00	0.9465
TF	0.16035	1	0.16035	0.03	0.8644
TS	2.98941	1	2.98941	0.55	0.4622
FS	5.86287	1	5.86287	1.08	0.3047
TFS	1.68640	1	1.68640	0.31	0.5803
ERROR	228.04000	42	5.42952		
DAY	209.94970	4	52.48742	15.12	0.0
DT	17.60019	4	4.40005	1.27	0.2879
DF	7.30110	4	1.82527	0.53	0.6771
DS	9.81244	4	2.45311	0.71	0.5585
DTF	2.33296	4	0.58324	0.17	0.9273
DTS	18.17862	4	4.54466	1.31	0.2734
DFS	3.45642	4	0.86411	0.25	0.8738
DTFS	31.53730	4	7.88433	2.27	0.0789
ERROR	583.13143	168	3.47102		

Median (+ lower and upper interquartile ranges) frequency and duration of bouts of *edgeon* behaviour exhibited by CBA pups exposed to aluminium sulphate (200mg/kg, Gd10-13).

FREQUENCY

Day	Cc	Ct	Tt	Tc
9	1.0 (0.0, 5.5)	1.0 (0.0, 4.0)	0.0 (0.0, 2.8)	1.0 (0.0, 2.0)

12	1.0 (0.0, 1.5)	2.0 (1.0, 4.0)	0.0 (0.0, 1.0)	0.0 (0.0, 1.0)
15	3.0 (2.0, 10.5)	8.0 (3.0, 16.0)	2.0 (0.3, 3.8)	2.0 (1.0, 7.5)
18	10.5 (2.0, 19.5)	11.0 (4.0, 19.0)	10.5 (5.0, 18.0)	11.5 (4.8, 19.0)

DURATION

Day	Cc	Ct	Tt	Tc
9	5.0 (0.0, 24.2)	3.1 (0.9, 16.3)	0.0 (0.0, 31.9)	3.1 (0.8, 16.3)
12	1.4 (0.0, 7.1)	0.0 (0.0, 1.9)	0.0 (0.0, 1.4)	0.0 (0.0, 1.9)
15	8.4 (1.9, 13.9)	1.7 (1.0, 9.1)	2.3 (0.3, 8.4)	1.7 (1.0, 9.1)
18	17.8 (4.0, 25.8)	16.3 (8.4, 20.1)	11.3 (4.3, 25.1)	16.3 (8.4, 20.1)

ANOVA table of CBA pup activity at weaning following prenatal exposure to aluminium sulphate (200mg/kg, Gd10-13).

SOURCE	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARES	F VALUE	TAIL PROB.
MEAN	151324.41211	1	151324.41211	548.97	0.0000
TREAT	13.31408	1	13.31408	0.05	0.8271
FMAT	104.38235	1	104.38235	0.38	0.5416
SEX	1149.63025	1	1149.63025	4.17	0.0474
TF	11.59804	1	11.59804	0.04	0.8385
TS	2729.43417	1	2729.43417	9.90	0.0030
FS	190.18522	1	190.18522	0.69	0.4109
TFS	392.67542	1	392.67542	1.42	0.2394
ERROR	11577.36190	42	275.62147		
MIN	1278.27969	4	319.56992	3.46	0.0140
MT	426.02479	4	106.50620	1.15	0.3320
MF	380.47507	4	95.11877	1.03	0.3871
MS	18.43235	4	4.60809	0.05	0.9908
MTF	324.47997	4	81.11999	0.08	0.4650
MTS	314.24608	4	78.56152	0.85	0.4805
MFS	387.82801	4	96.95700	1.05	0.3777
MTFS	225.73488	4	56.43372	0.61	0.6296
ERROR	15509.88571	168	92.32075		

CBA MALES

SOURCE	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARES	F VALUE	TAIL PROB.
MEAN	65957.23974	1	65957.23974	231.66	0.0000
TREAT	1235.23974	1	1235.23974	4.34	0.0491
FMAT	6.68150	1	6.68150	0.02	0.8796
TF	140.86612	1	140.86612	0.49	0.4892
ERROR	6263.70952	22	284.71407		
MIN	738.14212	4	184.53553	1.98	0.1287
MT	516.14212	4	129.03553	1.38	0.2567
MF	11.19121	4	2.79780	0.03	0.9916
MTF	14.85275	4	3.71319	0.04	0.9875
ERROR	8210.26667	88	93.29848		

CBA FEMALES

SOURCE	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARES	F VALUE	TAIL PROB.
MEAN	85648.08870	1	85648.08870	322.37	0.0000
TREAT	1496.00419	1	1496.00419	5.63	0.0278
FMAT	276.00419	1	276.00419	1.04	0.3203
TF	258.22954	1	258.22954	0.97	0.3360
ERROR	5313.65238	20	265.68262		
MIN	566.15748	4	141.53937	1.55	0.2046
MT	236.46734	4	59.11683	0.65	0.6056
MF	725.59410	4	181.39852	1.99	0.1165
MTF	513.36875	4	128.34219	1.41	0.2458
ERROR	7299.61905	80			

Median (+ lower and upper interquartile ranges) activity of CBA adult females at 22 weeks of age following prenatal exposure to aluminium sulphate (200mg/kg, Gd10-13).)

DAY 2

Minute	Cc	Ct	Tt	Tc
1	35.0 (27.3, 40.3)	21.0 (16.0, 37.3)	16.0 (11.0, 30.0)	25.0 (15.0, 46.5)
2	39.0 (34.3, 49.3)	24.0 (14.5, 47.7)	25.0 (13.0, 29.5)	29.0 (17.5, 33.0)
3	32.5 (27.5, 34.8)	19.5 (18.3, 34.3)	14.0 (9.0, 28.5)	33.0 (17.5, 38.5)
4	32.5 (22.3, 37.0)	18.0 (13.7, 51.2)	16.0 (12.5, 26.0)	26.0 (18.5, 30.5)
5	26.0 (21.8, 39.0)	21.0 (15.5, 49.0)	18.0 (7.0, 29.0)	15.0 (13.5, 34.0)

DAY 3

Minute	Cc	Ct	Tt	Tc
1	48.0 (43.5, 59.0)	25.5 (21.5, 41.5)	21.0 (14.5, 37.0)	33.0 (22.0, 41.5)
2	34.5 (33.5, 44.8)	26.0 (12.8, 38.5)	19.0 (14.5, 39.5)	29.0 (24.5, 37.0)
3	39.5 (28.5, 44.0)	16.5 (14.5, 33.3)	14.0 (10.5, 35.0)	20.0 (13.0, 25.5)
4	36.0 (26.0, 44.3)	19.5 (16.5, 29.5)	17.0 (8.0, 28.0)	29.0 (20.0, 33.0)
5	30.5 (23.3, 42.3)	21.5 (11.5, 46.5)	18.0 (11.0, 26.0)	21.0 (15.5, 33.0)

Median (+ lower and upper interquartile ranges) weight gained by CBA pups following prenatal exposure to aluminium sulphate (200mg/kg, Gd10-14).

Day	Cc	Ct	Tt	Tc
6	1.58 (1.48,1.64)	0.73 (0.66,1.56)	0.99 (0.63,1.07)	1.28 (1.16,1.37)
9	1.58 (1.52,1.70)	1.38 (1.35,1.42)	1.31 (1.20,1.39)	1.47 (1.36,1.50)
12	1.42 (0.92,1.51)	1.44 (1.32,1.55)	1.29 (1.29,1.41)	1.40 (1.23,1.42)
15	0.77 (0.54,0.90)	0.84 (0.73,1.50)	0.75 (0.72,1.19)	0.76 (0.51,1.09)
18	0.52 (0.41,0.83)	0.45 (0.33,0.61)	0.58 (0.52,0.70)	0.74 (0.40,1.00)
21	1.01 (0.76,1.13)	1.00 (0.87,1.12)	1.05 (0.58,1.15)	0.73 (0.56,0.94)

ANOVA table of the *crawling* behaviour of CBA pups prenatally exposed to aluminium sulphate (200mg/kg, Gd10-14).

FREQUENCY

SOURCE	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARES	F VALUE	TAIL PROB.
MEAN	12740.00196	1	12740.00196	454.83	0.0000
TREAT	461.22549	1	461.22549	16.47	0.0009
FMAT	0.07059	1	0.07059	0.00	0.9606
SEX	33.64902	1	33.64902	1.20	0.2893
TF	8.54118	1	8.54118	0.30	0.5884
TS	14.16667	1	14.16667	0.51	0.4872
FS	5.71765	1	5.71765	0.20	0.6575
TFS	15.88235	1	15.88235	0.57	0.4624
ERROR	448.16667	16	28.01042		
DAY	7102.07157	4	1775.51789	78.21	0.0000
DT	275.00490	4	68.75123	3.03	0.0392
DF	168.37549	4	42.09387	1.85	0.1510
DS	29.16961	4	7.29240	0.32	0.8071
DTF	106.80686	4	26.70172	1.18	0.3285
DTS	21.31863	4	5.32966	0.23	0.8691
DFS	87.98333	4	21.99583	0.97	0.4143
DTFS	92.79902	4	23.19975	1.02	0.3906
ERROR	1453.00000	64	22.70312		

DURATION

SOURCE	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARES	F VALUE	TAIL PROB.
MEAN	279876.04406	1	279876.04406	451.59	0.0000
TREAT	8083.77866	1	8083.77866	13.04	0.0023
FMAT	962.84414	1	962.84414	1.55	0.2305
SEX	3480.69892	1	3480.69892	5.62	0.0307
TF	100.54824	1	100.54824	0.16	0.6924
TS	260.43879	1	260.43879	0.42	0.5260
FS	86.51173	1	86.51173	0.14	0.7136
TFS	613.91406	1	613.91406	0.99	0.3344
ERROR	9916.16674	16	619.76042		
DAY	123382.54568	4	30845.63642	41.65	0.0000
DT	3432.61103	4	858.15276	1.16	0.3351
DF	1142.87824	4	285.71956	0.39	0.7627
DS	4265.52257	4	1066.38064	1.44	0.2429

DTF	916.67771	4	229.16943	0.31	0.8174
DTS	627.90575	4	156.97644	0.21	0.8867
DFS	1567.19515	4	391.79879	0.53	0.6636
DTFS	3498.18383	4	874.54596	1.18	0.3268
ERROR	47392.58375	64	740.50912		

HEADUP

FREQUENCY

SOURCE	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARES	F VALUE	TAIL PROB.
MEAN	13895.26628	1	13895.26628	1009.81	0.0000
TREAT	4.20840	1	4.20840	0.31	0.5874
FMAT	5.84912	1	5.84912	0.43	0.5231
SEX	35.31020	1	35.31020	2.57	0.1276
TF	4.37740	1	4.37740	0.32	0.5801
TS	20.23634	1	20.23634	1.47	0.2418
FS	0.12590	1	0.12590	0.01	0.9249
TFS	5.08664	1	5.08664	0.37	0.5512
ERROR	233.92500	17	13.76029		
DAY	3991.09270	5	798.21754	68.50	0.0000
DT	108.46196	5	21.69239	1.86	0.1457
DF	23.26435	5	4.65287	0.40	0.7605
DS	52.47394	5	10.49479	0.90	0.4499
DTF	166.48059	5	33.29612	2.86	0.0441
DTS	10.72144	5	2.14429	0.18	0.9119
DFS	78.10933	5	15.62187	1.34	0.2709
DTFS	24.33821	5	4.86764	0.42	0.7474
ERROR	990.55833	85			

DURATION

SOURCE	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARES	F VALUE	TAIL PROB.
MEAN	250735.05868	1	250735.05868	1051.77	0.0000
TREAT	2080.16431	1	2080.16431	8.73	0.0089
FMAT	14.60361	1	14.60361	0.06	0.8075
SEX	209.03236	1	209.03236	0.88	0.3622
TF	28.58817	1	28.58817	0.12	0.7334
TS	11.04833	1	11.04833	0.05	0.8321
FS	13.38338	1	13.38338	0.06	0.8155
TFS	103.04587	1	103.04587	0.43	0.5197
ERROR	4052.69164	17	283.39363		
DAY	66735.61036	5	13347.12207	25.45	0.0000
DT	4657.18827	5	931.43765	1.78	0.1771
DF	285.31141	5	57.06228	0.11	0.9234
DS	1329.56111	5	265.91222	0.51	0.6356
DTF	3123.77807	5	624.75561	1.19	0.3195
DTS	740.50779	5	148.10156	0.28	0.7897
DFS	1504.01321	5	300.80264	0.57	0.5951
DTFS	2454.68530	5	490.93706	0.94	0.4136
ERROR	44573.13883	85	524.38987		

REARING

FREQUENCY

SOURCE	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARES	F VALUE	TAIL PROB.
MEAN	3871.42061	1	3871.42061	206.32	0.0000
TREAT	127.07905	1	127.07905	6.77	0.0180
FMAT	58.40278	1	58.40278	3.11	0.0947
SEX	6.79098	1	6.79098	0.36	0.5549
TF	6.79098	1	6.79098	0.36	0.7243
FS	7.73748	1	7.73748	0.41	0.5289
TFS	0.00772	1	0.00772	0.00	0.9840
ERROR	337.75000	18	18.76389		
DAY	1470.20460	2	735.10230	45.71	0.0
DT	84.71448	2	42.35744	2.63	0.0896
DF	152.59691	2	76.29846	4.74	0.0169
DS	4.49678	2	2.24839	0.14	0.8575
DTF	8.49266	2	4.24633	0.26	0.7556
DTS	15.05645	2	7.52822	0.47	0.6180
DFS	49.01529	2	24.50765	1.52	0.2328
DTFS	0.75741	2	0.37870	0.02	0.9717
ERROR	578.90000	36	16.08056		

STILL

SOURCE	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARES	F VALUE	TAIL PROB.
MEAN	944.79840	1	944.79840	335.31	0.0000
TREAT	22.42715	1	22.42715	7.96	0.0118
FMAT	0.96607	1	0.96607	0.34	0.5659
SEX	2.30739	1	2.30739	0.82	0.3781
TF	0.0	1	0.0	0.0	1.0000
TS	0.03194	1	0.03194	0.01	0.9165
FS	0.28743	1	0.28743	0.10	0.7533
TFS	4.22355	1	4.22355	1.5	0.2375
ERROR	47.90000	17	2.81765		
DAY	19.09242	4	4.77310	1.27	0.2940
DT	28.11038	4	7.02759	1.87	0.1455
DF	47.13234	4	11.78308	3.14	0.0327
DS	5.40379	4	1.35095	0.36	0.7840
DTF	11.10439	4	2.77610	0.74	0.5345
DTS	11.95469	4	2.98867	0.80	0.5027
DFS	5.35589	4	1.33897	0.36	0.7863
DTFS	13.07246	4	3.26811	0.87	0.4633
ERROR	255.30000	68	3.75441		

Median (+ lower and upper interquartile ranges) number of trials required by CBA males to enter all 8 arms in an 8-arm radial maze, following prenatal exposure to aluminium sulphate (200mg/kg, Gd10-14).

NUMBER OF TRIALS

Trial	Control	Treated
1	14.0 (12.3, 16.0)	16.0 (12.0, 16.0)
2	11.5 (8.8, 16.0)	15.0 (12.0, 16.0)
3	8.5 (8.0, 12.0)	14.0 (10.0, 15.5)
4	8.0 (8.0, 10.0)	15.0 (10.0, 16.0)
5	8.5 (8.0, 16.0)	10.0 (8.0, 15.0)
6	10.5 (8.0, 13.5)	8.0 (8.0, 15.5)
7	9.0 (8.0, 11.0)	10.0 (8.5, 12.5)
8	13.0 (8.0, 16.0)	8.0 (8.0, 12.5)
9	10.0 (8.0, 12.3)	10.0 (8.0, 12.0)
10	8.5 (8.0, 12.3)	8.0 (8.0, 8.0)
11	8.5 (8.0, 11.5)	8.0 (8.0, 11.5)
12	9.0 (8.0, 13.0)	10.0 (8.5, 14.0)

CORRECT RESPONSES

Trial	Control	Treated
1	5.5 (5.0, 7.0)	6.0 (5.5, 7.0)
2	7.0 (6.8, 7.3)	6.0 (5.0, 7.0)
3	7.5 (7.0, 8.0)	6.0 (5.5, 7.0)
4	8.0 (7.5, 8.0)	6.0 (4.5, 7.5)
5	7.5 (7.0, 8.0)	7.0 (5.5, 8.0)
6	7.0 (7.0, 8.0)	8.0 (5.5, 8.0)
7	7.5 (6.8, 8.0)	7.0 (7.0, 7.5)
8	7.0 (6.0, 8.0)	8.0 (7.5, 8.0)
9	7.0 (6.0, 8.0)	7.0 (6.5, 8.0)
10	7.5 (6.8, 8.0)	8.0 (8.0, 8.0)
11	7.5 (6.8, 8.0)	8.0 (6.5, 8.0)
12	7.5 (6.8, 8.0)	7.0 (6.5, 7.5)

APPENDIX C

Alterations to the Pattern of Ultrasonic Calling after Prenatal Exposure to Aluminium Sulfate

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Pregnant CBA mice were exposed to aluminium sulfate at a dose of 200 mg/kg body wt injected intraperitoneally during Days 10 to 13 of gestation. We used a variety of ethological measures, which have been shown to be sensitive indicators of toxicants, to assess effects on the mother and the behavioral development of pups. Prenatal aluminium resulted in a reduction in the rate of ultrasonic calling by pups accompanied by a shift in the timing of peak calling; treated pups exhibited decreased growth and delays in neurobehavioral development. The treatment received by a pup's foster mother was also found to influence development. We recommend ultrasonic calling as a sensitive measure in studies of behavioral teratogenicity.

The production of ultrasounds by the young of many rodent species is now well established (e.g., Okon, 1972; Sales & Smith, 1978; Elwood & McCauley, 1983) and its function as a form of communication between young and mother has been characterized. Calls may be elicited as a result of gentle handling (Okon, 1970b), during isolation (Noirot, 1966; Robinson & D'Udine, 1982), under cold stress (Okon, 1970a), or from exposure to unusual tactile (Okon, 1970b) or olfactory stimuli (Oswalt & Meier, 1975).

Rodent neonates emit ultrasonic calls with a reliable and stable pattern from a few days after birth. Their calling is easily measured and thus this response could form a marker against which the results of environmental or physiological changes can be measured. As pointed out by Zbinden (1981), this response may function as a sensitive indicator of subtle behavioral effects caused by prenatal or postnatal drug treatments. As yet, few studies have incorporated this technique in their test battery aimed at assessing the behavioral teratogenicity of an agent despite the recommendation by Cuomo, DeSaliva, Maselli, Santo, and Cagiano (1987). In those studies which have measured ultrasounds both increases and decreases in calling have been described. Rat pups were treated chronically with haloperidol, a dopamine antagonist, during the early postnatal period (Days 2–16) which resulted in a reduction in the rate and frequency of their ultrasonic calls when isolated (Cagiano, Sales, Renna, Racagni, & Cuomo, 1986). Similarly, Adams (1982) recorded ultrasonic calls from Sprague–Dawley rats treated prenatally with vitamin A palmitate, a known behavioral teratogen. Pups exposed at the highest dose level (30,000 IU/kg) called at a significantly higher rate than controls.

As part of our studies on behavioral teratology we have reported earlier a marked effect of the treatment received by a pup's foster mother during gestation on aspects of its physical and behavioral development (Clayton, Sedowofia, Rankin, & Manning, 1993). Since ultrasonic calling forms part of the complex intercommunication system between a mother and her litter, we have investigated the pattern of ultrasonic emissions in treated and control mice in an attempt to elucidate some of the mechanisms which might be implicated. In this case we treated pregnant mice with aluminium.

METHODS

Treatment

CBA/T6 virgin females between 10 and 12 weeks of age were mated with males from the same strain. Plugged females were individually housed in standard plastic cages (310 × 130 × 120 cm) lined with wood shavings. All mice were maintained in an air-

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cycle (lights on 2100 h) at a temperature of 22–23°C. On Gestational Day 10 (GD10) pregnant females were randomly assigned to one of two groups. The groups were coded such that the experimenter was blind to the allocation of treatment groups. Over GD10 to 13 inclusive the females of one group were administered aluminium sulfate intraperitoneally (ip) at a dose of 200 mg/kg body wt and the control group received the equivalent volume of saline acidified to the same pH as the aluminium solution (pH 4.1). Maternal weights were recorded on Days 10, 12, and 18 of gestation.

Procedure

Pregnant females were checked twice daily for pups. At birth, litter size was recorded and the sex of each pup determined. On Postnatal Day 1 (Pd1), all pups born were weighed and each litter was culled to four pups, leaving two males and two females where possible. Each pup was toe-clipped for individual identification. The pups were cross-fostered using a balanced, split-litter total cross-foster procedure (Sedowofia, Innes, Alleva, Manning, & Clayton, 1989) with other litters born within the same 12-h period. Briefly, each mother received two control and two treated pups from other litters. In this way none of the mothers reared any of their own pups. Thus, the pups could be classified into four treatment groups; control pups cross-fostered to control mothers (Cc), control pups fostered to treated mothers (Ct), treated pups fostered to control mothers (Tc), and treated pups fostered to treated mothers (Tt).

Ultrasonic Recording

Ultrasonic emissions were recorded on 6 days (on PD3, 4, 5, 6, 9, and 12). These days were chosen on the basis of pilot studies and from previous work which typically showed that few calls are emitted at birth and that CBA pups have ceased calling by Days 12–13.

The litter was removed from the home cage and placed in a crystalizing dish lined with nesting material (dish 1), and kept at a temperature comparable to that of the maternal nest (34–36°C) with a lamp (25 W). Each pup was then removed individually from dish 1 and placed in a second dish (dish 2) which was cooler (24–26°C) and acted as a mild thermal stressor. Ultrasounds were recorded using a QMC Instruments Mini-2 Bat detector set at a frequency of 70–80 kHz (see Robinson & D'Udine, 1982) and mounted 10 cm above dish 2. minute was counted for 5 min. Scoring was delayed for 1 min after placing the pup in dish 2 to allow any immediate response to handling to die down. The same type of nesting material as used for the maternal nest was placed in each dish to reduce sound emissions due to unusual tactile stimuli (Noirot & Pye, 1969; Okon, 1970b).

Recording for 5 min was sufficient to even out short-term fluctuations in calling. It was not uncommon for pups to produce calls at a rate greater than 250/min. In these instances our method of recording may have underestimated the number of calls produced, although this would have been constant across all treatment groups. The pup was replaced into dish 1 and the procedure was repeated for each pup in the litter. Individual pups were not returned to the home cage until all pups in the litter had been tested. The order in which litters and individual pups were recorded was randomized on each test day.

Body weight was recorded every third day from Pd3 to weaning at Pd21. At the same time pups underwent the modified version of the Fox tests for sensorimotor coordination (Fox, 1965) as reported for an earlier study (Sedowofia et al., 1989).

Maternal Retrieval

The maternal retrieval test was similar to that employed by Noirot (1966). On completion of recording of ultrasonic calling, the four pups were replaced together into the home cage at the opposite end from the maternal nest, waiting until the mother had moved into it. The time taken to return one of the pups to the nest was recorded to the nearest second.

Data Analysis

Maternal weight gain during gestation and pup birth weight were compared using Student's t test. Thereafter, body weights were analyzed using analysis of variance (ANOVA) with repeated measures; prenatal treatment, foster mother treatment, and pup sex were the independent factors and day was the repeated measure (Dixon, 1988). Because of variability within the groups and the occurrence of zero scores, the ultrasonic data were analyzed using the Kruskal–Wallis one-way ANOVA which includes a multiple comparisons option (Dixon, 1988). The Fox test scores were analyzed by χ^2 tests (Ryan, Joiner, & Ryan, 1979).

RESULTS

Maternal Weights

Prenatal exposure to ip aluminium sulfate during Gestation Days 10–13 had no effect on breeding performance. The mean gestation length for control mothers (n = 10) was 19.9 ($\pm$0.23) and 20 ($\pm$0.37) days for those females exposed to aluminium (n = 9). Pup mortality occurred in both treatment groups. Although control females were found to have a larger mean litter size (x = 6.0 $\pm$ 0.71) compared to aluminium exposed females (x = 4.8 $\pm$ 0.8), this difference was not significant. There were no differences in the amount of food or water consumed by control and treated females during pregnancy or the preweaning period.

Weight gain during pregnancy was adversely affected by exposure to aluminium, treated mothers gaining significantly less weight during Gestation Days 10–18 than control mothers (control: x = 8.69 ($\pm$0.7) g; treated: x = 5.65 ($\pm$0.81) g, $t(16) = 2.84$, $p < .02$). However, maternal weights during lactation up until weaning were largely unaffected by treatment.

Pup Weights

There was a highly significant difference between mean birth weights (control: n = 37, x = 1.35 $\pm$ 0.02 g; treated: n = 25, x = 1.19 $\pm$ 0.03 g, $t(37) = 3.95$, $p = .0003$), pups exposed to aluminium were 12% lighter than controls.

ANOVA revealed a highly significant effect of prenatal ($F(1, 33) = 32.34$, $p < .001$) and foster mother treatment ($F(1, 33) = 54.56$, $p < .001$) on pup weight during the preweaning period. As can be seen from Fig. 1, Cc pups were found to be the heaviest group and Tt pups weighed the least, a difference of 20% by Pd21. The treatment groups ran parallel to their foster mother treatment; those pups fostered to control mothers weighed more than pups reared by mothers who had been exposed to aluminium. For example, by the time of weaning Ct pups weighed 11% less than their control counterparts (Cc) and similarly Tt pups were 14% lighter than Tc pups.

Fox Tests

χ^2 analysis of the seven Fox tests used revealed a highly significant difference between the treatment groups in forelimb grasping on Pd12 ($\chi^2(3) = 20.401$, $p < .001$) with only two out of nine Tt pups having reached an adult response by this age and in screen climbing on Pd15 ($\chi^2(3) = 11.16$, $.05 > p > .02$). Performance in the other tests was not significantly different between the groups.

Ultrasonic Calling

Figure 2 summarizes the changes in the total number of ultrasounds emitted on each test day by the four treatment groups as a function of age. As expected calls were more frequent during the earliest postnatal days reaching a peak which then steadily declined such that by Day 12 very little calling was evoked by the drop in temperature. It is not possible to say when calling ceased completely as no further measurements were taken after Pd12.

The most striking result is that, throughout the test period, the total number of calls emitted was dramatically reduced in those pups exposed to aluminium in utero. Kruskal–Wallis one-way ANOVA showed this difference to be especially pronounced on Days 3 ($H(3) = 12.79$, $p < .006$) and 4($H(3) = 20.95$, $p = .001$). The rate of calling of Cc pups increased to a peak on Postnatal Day 4 (a median rate of 204.5 calls/min) and then slowly declined to a median of less than 2 calls/min by Pd12. Ct and Tc pups produced their greatest number of calls on Day 5 (196.5 and 114.5 calls/min, respectively) and Tt pups on Day 6 (128 calls/min). Although control pups from both groups emitted more total calls than treated pups, Ct pups had a considerably lower initial rate of calling on Day 3. Their rate of output reached levels comparable to that of Cc pups only on Day 5. The decline in calling with age was also more dramatic for this group. Thus, being raised by a treated mother dampened the rate at which these pups called. In summary, prenatal aluminium exposure resulted in a reduction in the rate of calling and shifted the timing of peak calling.

Maternal Retrieval

Although control mothers had a shorter latency to retrieve on Pd3 there was no effect of treatment on the time taken to first retrieval. As pups grew older both groups tended to reduce their latency to contact the pup. Maternal retrieval was not tested on Pd12 as pups were capable of homing to the nest before the mother could pick them up.

DISCUSSION

Some of the trends recorded in this experiment are in agreement with those previously reported (Clayton et al., 1993) where aluminium administered to a pup's foster mother during gestation was

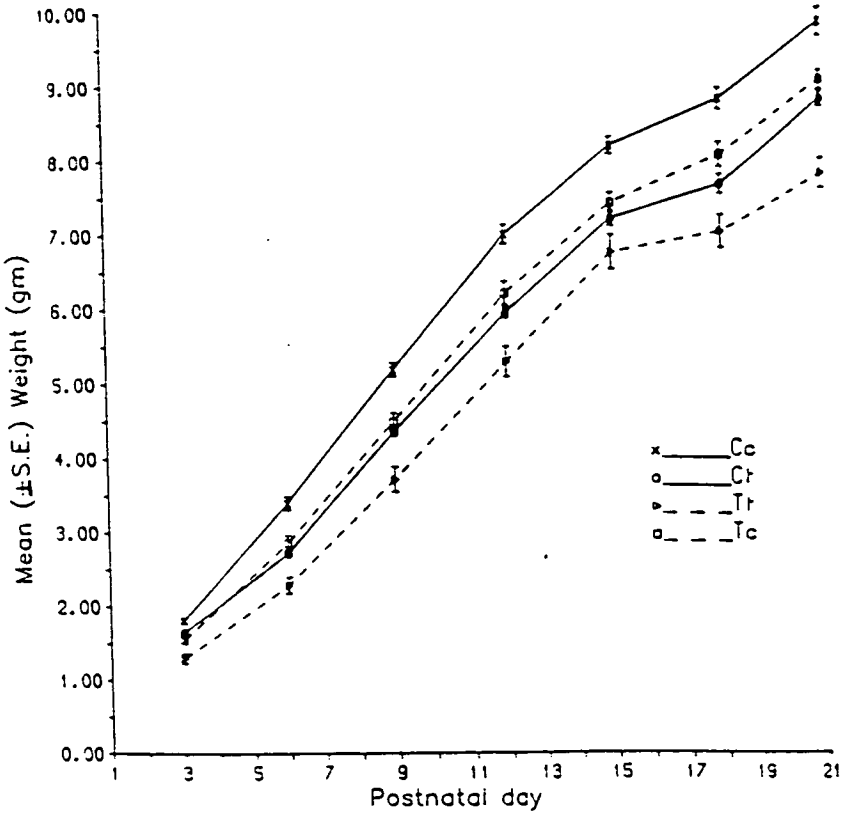

FIG. 1. CBA pup body weights of offspring whose mothers received intraperitoneal injections of either aluminum sulphate or saline during gestation days 10 to 13. Weights were recorded every third day from birth to weaning.

found to have a marked effect on pup body weights up to weaning. By contrast, our results show a direct effect on a pup's production of ultrasounds which is strongly reduced by exposure to aluminium *in utero*. It is not possible to determine with confidence whether this disparity in calls was due to effects of exposure to aluminium on the vocalization system per se or on its rate of maturation. It is unlikely to be a result of damaged vocal apparatus as pups from all groups appeared to call normally when they did so. However, treated pup calling did not reach a maximum until a day later than control pups. It would certainly seem possible that the mechanisms of sound production matured more rapidly in control pups.

The results of the sensorimotor tests imply that pups exposed to aluminium prenatally were, in some respects, slower to mature than control pups. Similar delays in neurobehavioral development following exposure to Al have been reported previously (Bernuzzi, Desor, & Lehr, 1986, 1989; Donald, Golub, & Gershwin, 1989). This finding also gives some support to the idea that the differential profiles of calling for the treatment groups may have resulted from a delay in the maturation of the mechanisms responsible for ultrasonic vocalizations.

However, other possible explanations must be considered. The differential calling profiles may be related to changes in the pups' detection of or response to chilling. As mentioned previously several investigators have established a correlation between the emission of ultrasonic calls and the development of homiothermy (Okon 1970a; Allin & Banks, 1971). The typical age profile for calling when exposed to cooling is one of few calls immediately after birth during the period of poikilothermy, followed by a greater intensity of calling during the first stages of development of thermoregulatory ability, and finally a decrease in the rate of calling as thermoregulation is achieved. This is the calling profile followed by our Cc pups, whereas treated pups did not exhibit these characteristic changes with age. Exposure to aluminium might lead to a prolongation of the early stage of poikilothermy. To eliminate this possibility would require investigations into the processes underlying temperature regulation.

Ultrasounds certainly function to elicit maternal

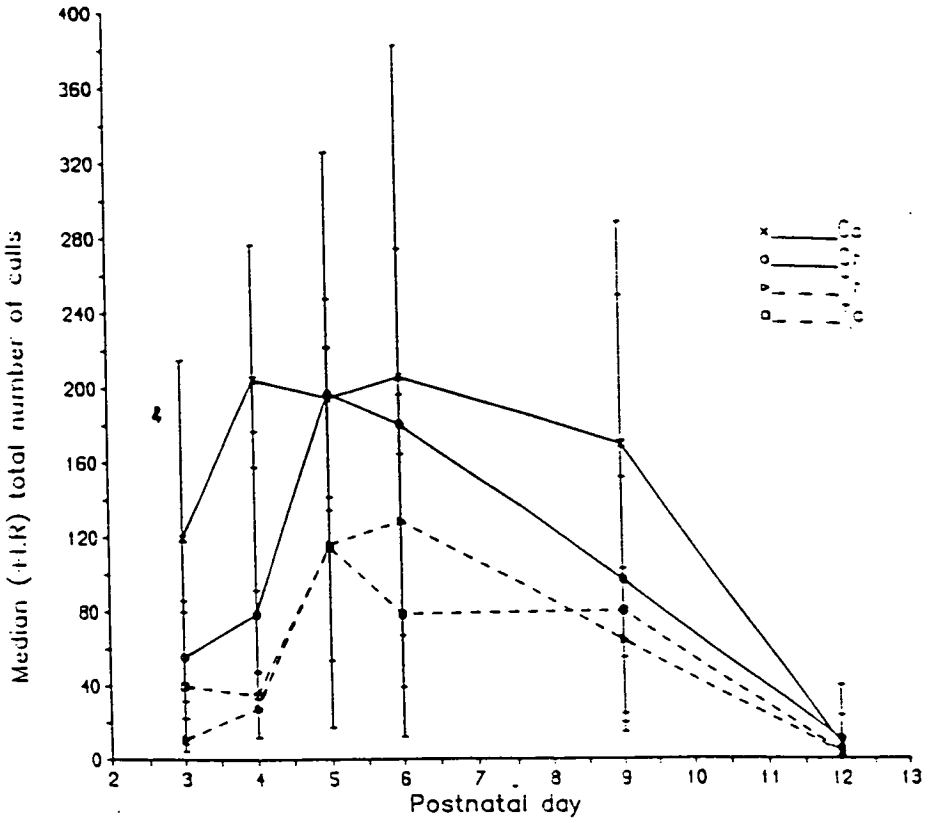

FIG. 2. The total number of CBA pup ultrasonic calls emitted in a 5-min test for each of the four treatment groups on each of 6 test days.

responses, particularly evoking searching by the mother and retrieval of lost pups back to the security of the nest. If pups exhibit weak or fewer calls this might have serious repercussions for survival of the litter.

However, despite the altered level and pattern of calling in treated pups, our results do not reveal any change to the mother's responses. This is not surprising, for three reasons. First, although the procedure used to test maternal retrieval was that employed in many other studies, it may not be a good discriminator between treatment groups, as the distance over which the females had to travel was small. Second, some mothers were seen to be having difficulty seizing a pup as it was moving especially during the latter test days. This meant a lengthened time to retrieve, although the mother may have responded just as quickly. Finally, Beach and Jaynes (1956) have described a number of stimuli to which mother rats respond when retrieving their pups. For example, they showed that if the olfactory characteristics of a pup are altered (by covering it with perfume) the pup is still eventually retrieved, although control pups are retrieved preferentially. Thus, even if aluminium affected one aspect of the pup's stimulus quality, other sensory modalities would remain to evoke a normal reaction. In addition, as mothers were tested for retrieval with whole litters, the two control pups could provide a normal ultrasound stimulus. It will be interesting to investigate whether mothers retrieve control pups first in this situation.

Since there was no difference in the amount of food or water ingested by control and treated mothers, the body weight differences of their pups could not have resulted from undernourishment of the treated mothers which has been shown to result in less efficient retrieval of young and in a reduction in licking (Smart & Preece, 1973).

Certainly alterations in maternal behavior are known to affect infant development and several drugs have been shown to disrupt elements of maternal behavior (Laviola, Sedowofia, Innes, Clayton, & Manning, 1990). Thus, any interruption to the pattern of maternal care might explain why Ct pups, who were found to emit a similar profile of calls although at a lower level compared to their Cc counterparts, had body weights notably reduced from that of Cc pups. Similarly, Tc and Tt pups had comparable profiles of calling but Tc pups were of

greater body weight. In the case of Tt pups the diminished number of calls may have affected the ability of the treated mother to provide adequate maternal responses even in the presence of calls from control pups. Tc pups had a similar reduced profile of calls to that of Tt pups, the difference being that these treated pups were raised by control mothers. These mothers may have been more finely tuned to their pups such that even a diminution in the number of calls was not sufficient to alter maternal responsiveness. It will be necessary to investigate further the maternal behavior of mothers rearing control and aluminium-treated pups.

Terkel, Damassa, and Sawyer (1979) claim that ultrasonic calling stimulates the release of prolactin from the mother's anterior pituitary (although Stern, Thomas, Rabii, & Barfield, 1984, do not confirm this). The primary stimulus for prolactin secretion is probably suckling although several other features of the pup have been found to increase its release. If ultrasounds are indeed one such feature, then the increase in sound emissions by Cc pups could have the effect of augmenting the nourishment available for them, which has obvious consequences for growth.

The exact mechanisms underlying the regulation of ultrasonic calling are not known. The development of the vocalization system is undoubtedly complex and probably involves several neurotransmitter systems. However, Brudzynski and Bihari (1990) have suggested the involvement of the cholinergic system in their production. In a previous paper (Clayton et al., 1993) we reported a reduction in choline acetyltransferase activity in brains of mice exposed to aluminium *in utero*. That such treatment led to a reduction in calling is at least consistent with Brudzynski and Bihari's (1990) hypothesis.

In summary, prenatal exposure to aluminium causes alterations to growth, ultrasound production, and sensorimotor development. Additional work is required to investigate the consequences of a lowered calling rate on the mother–infant interaction but the results we report here certainly suggest that ultrasound recording should be included in any battery of tests aimed at assessing possible behavioral teratogenicity.

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APPENDIX D

ANOVA table of CBA maternal body weight following gestational exposure to aluminium sulphate (200mg/kg, Gd10-13).

SOURCE	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARES	F VALUE	TAIL PROB.
MEAN	25730.25654	1	25730.25654	1181.45	0.0000
TREAT	22.05225	1	22.05225	1.01	0.3438
ERROR	174.22900	8	21.77863		
DAY	10.21275	3	3.40425	19.57	0.0004
DT	5.56475	3	1.85492	10.66	0.0040
ERROR	4.17500	24	0.17396		

ANOVA table of CBA pup body weights following prenatal exposure to aluminium sulphate (200mg/kg, Gd10-13).

SOURCE	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARES	F VALUE	TAIL PROB.
MEAN	5673.06940	1	5673.06940	7462.77	0.0000
TREAT	23.36847	1	23.36847	30.74	0.0000
FMAT	23.06733	1	23.06733	30.34	0.0000
SEX	0.09565	1	0.09565	0.13	0.7254
TF	0.83946	1	0.83946	1.10	0.3020
TS	0.07811	1	0.07811	0.10	0.7508
FS	2.04557	1	2.04557	2.69	0.1117
TFS	2.15447	1	2.15447	2.83	0.1030
ERROR	22.04530	29	0.76018		
DAY	1172.61478	6	195.43580	3076.22	0.0000
DT	1.75672	6	0.29279	4.61	0.0032
DF	3.38538	6	0.56423	8.88	0.0000
DS	0.10724	6	0.01787	0.28	0.8623
DTF	0.29221	6	0.04870	0.77	0.5305
DTS	0.04774	6	0.00796	0.13	0.9590
DFS	0.22432	6	0.03739	0.59	0.6451
DTFS	0.22199	6	0.03700	0.58	0.6493
ERROR	11.05443	174	0.06353		

C57BL/6J

SOURCE	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARES	F VALUE	TAIL PROB.
MEAN	15824.29810	1	15824.29810	9018.72	0.0000
TREAT	4.23017	1	4.23017	2.41	0.1272
FMAT	21.80863	1	21.80863	12.43	0.0010
SEX	10.23070	1	10.23070	5.83	0.0197
TF	0.42728	1	0.42728	0.24	0.6240
TS	1.49818	1	1.49818	0.85	0.3602
FS	0.41257	1	0.41257	0.24	0.6300
TFS	0.32710	1	0.32710	0.19	0.6679
ERROR	82.46647	47	1.75461		

DAY	2384.07038	6	397.34506	3699.91	0.0000
DT	1.33152	6	0.22192	2.07	0.1308
DF	4.85441	6	0.80907	7.53	0.0008
DS	2.30723	6	0.38454	3.58	0.0304
DTF	0.29096	6	0.04849	0.45	0.6435
DTS	0.55542	6	0.09257	0.86	0.4282
DFS	0.88719	6	0.14786	1.38	0.2573
DTFS	0.07437	6	0.01239	0.12	0.8961
ERROR	30.28491	282	0.10739		

MALES

SOURCE	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARES	F VALUE	TAIL PROB.
MEAN	9251.42251	1	9251.42251	4953.35	0.0000
TREAT	0.38556	1	0.38556	0.21	0.6533
FMAT	9.01943	1	9.01943	4.83	0.0371
TF	0.83516	1	0.83516	0.45	0.5096
ERROR	48.56048	26	1.86771		
DAY	1403.71388	6	233.95231	2407.51	0.0000
DT	0.17700	6	0.02950	0.30	0.7502
DF	1.75381	6	0.29230	3.01	0.0551
DTF	0.12235	6	0.02039	0.21	0.9733
ERROR	15.15945	156	0.09718		

FEMALES

SOURCE	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARES	F VALUE	TAIL PROB.
MEAN	6827.26595	1	6827.26595	4228.53	0.0000
TREAT	4.88919	1	4.88919	3.03	0.0965
FMAT	12.81907	1	12.81907	7.94	0.0103
TF	0.00303	1	0.00303	0.00	0.9658
ERROR	33.90600	21	1.61457		
DAY	1021.19119	6	170.19853	1417.81	0.0000
DT	1.56967	6	0.26161	2.18	0.1279
DF	3.78337	6	0.63056	5.25	0.0101
DTF	0.23194	6	0.03866	0.32	0.7184
ERROR	15.12546	126	0.12004		

Median (+ lower and upper interquartile ranges) number of ultrasonic calls produced by CBA pups prenatally exposed to aluminium sulphate (200mg/kg, Gd10-13).

Day	Cc	Ct	Tt	Tc
3	119.5 (41.7, 215.2)	55.5 (31.7, 86.2)	11.0 (4.5, 22.5)	40.0 (8.5, 80.2)
4	204.5 (157.5, 276.5)	79.0 (47.2,153.5)	28.0 (12.0, 48.5)	35.0 (26.5, 92.0)
5	194.0 (141.0, 325.5)	196.5 (134.0, 220.7)	116.0 (53.5, 221.5)	114.5 (17.2, 247.2)
6	205.0 (163.5, 273.5)	179.5 (66.5, 382.2)	128.0 (39.0 204.5)	78.0 (12.0, 195.7)
9	169.0 (14.5, 248.7)	96.5 (55.0, 151.5)	64.0 (24.5, 288.0)	80.0 (19.7, 102.7)
12	7.5 (0.0, 40.2)	10.5 (0.8, 23.8)	3.0 (1.5, 10.5)	3.5 (2.0, 7.3)

C57BL/6J

Day	Cc	Ct	Tt	Tc
3	41.0 (15.5, 82.5)	23.0 (9.5, 60.5)	36.0 (10.8, 64.3)	78.0 (12.0, 188.0)
4	16.0 (8.3, 27.8)	30.0 (10.0, 93.7)	21.0 (14.5, 48.5)	11.0 (6.0, 39.0)
6	22.5 (9.5, 71.0)	17.5 (8.0, 29.0)	53.0 (14.5, 90.7)	54.0 (10.0, 77.0)
8	76.5 (25.0, 109.8)	29.5 (20.0, 76.7)	30.5 (20.0, 68.0)	75.0 (51.0, 124.0)

Median (+ lower and upper interquartile ranges) latency (s) to first retrieval of a pup back to the nest by CBA mothers exposed to aluminium sulphate (200mg/kg, Gd10-13) or saline by i.p. injection.

Day	Control	Treated
3	24.0 (18.5, 36.5)	54.0 (29.0,112.0)
4	21.0 (18.0, 50.0)	32.0 (25.5, 42.5)
5	19.0 (15.5, 32.0)	29.0 (26.0, 34.5)
6	40.0 (25.5, 42.0)	25.0 (21.5, 41.0)
9	26.0 (17.0, 32.2)	17.0 (16.0, 56.0)

APPENDIX E

Rankin, J., Laviola, G., Valanzano, A., Alleva, E. and Manning, A.

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EFFECT OF GESTATIONAL EXPOSURE TO ALUMINIUM SULPHATE ON MOUSE MATERNAL BEHAVIOR.

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ABSTRACT

Pregnant CBA and C57BL/6J females were exposed to aluminium sulphate at a dose of 200mg/kg by intraperitoneal injection during gestation days 10-13 inclusive. The offspring were cross-fostered at birth, and maternal behavior was observed during the first 3 postpartum weeks. Aluminium caused a slight reduction in maternal weight gain during gestation. Only moderate changes in maternal behavior were observed. Al-exposed CBA females spent less time nursing and were more involved in non pup-directed behaviors. Conversely, C57 females showed enhanced nursing. Previously reported alterations to pup development are likely to have resulted from a direct effect of Al rather than any disruption to maternal behavior.

GESTATIONAL ALUMINIUM AND MATERNAL BEHAVIOR

ALUMINIUM GESTATION MATERNAL BEHAVIOR INBRED MICE

INTRODUCTION

The maintenance of maternal care in rodents depends not only on hormonal influences (Rosenblatt and Siegel, 1983) but also on the efficiency of the bidirectional exchange of a number of sensory cues between the mother and her offspring, both playing an important active role (Smith and Sales, 1980). For example, ultrasonic vocalizations emitted by pups play an important communicative role in this relationship, specifically as directional cues (Noirot, 1972). Further, olfactory stimuli are important in pup recognition as evidenced from lesions of the noradrenergic projection to the olfactory bulbs in nulliparous BALB/c females before parturition which resulted in cannibalistic behavior (Dickinson and Keverne, 1988).

More specifically, maternal behavior is sensitive to subtle stimulus changes which can alter this behavior and in turn may have longterm effects on the development of the offspring. It has been suggested that the enhancing effects of early handling procedures on pup development may be explained in terms of changes in MB. For example, several studies have described increased licking behavior by mothers following handling of young (Lee and Williams, 1974; Priestnall 1983). Certain characteristics of the litter have also been shown to modify maternal behavior (Alleva, *et al.*, 1989).

Recently, studies involving the investigation of the effects of administration of drugs or toxicants during pregnancy on offspring development, have examined the effect of such treatments on the expression and pattern of maternal behavior. Alcohol-exposed CFW females were found to exhibit an increase in the frequency of exploratory behavior 1-day after birth (Ewart and Cutler, 1979). Interestingly, Hard and colleagues (1985) found that female Wistar rats, exposed *in utero* to ethanol (16% ethanol solution), took longer to retrieve their pups and built nests of a lower depth than controls.

By contrast, enhancements of maternal behavior have also been reported. Outbred female mice exhibited an increased duration of time involved in nursing and an increased frequency and duration of social investigation of their young on Pd1 following exposure to phenobarbitone (PhB) in their drinking water (120-190mg/kg daily) throughout gestation and lactation (Chapman and Cutler, 1983). At Pd21 PhB-treated females were still actively engaged in nursing behavior (a mean duration of 61s for treated females compared to a mean of 0.4s for the control group). Futhermore, Laviola and colleagues (1990) have demonstrated strain-specific differences in the influence of prenatal exposure to PhB (60mg/kg by i.p. injections during days 10-16 of gestation) on maternal behaviour. CBA treated females continued to nurse their offspring for longer than controls whilst C57 PhB-exposed dam's exhibited less nursing behavior.

These studies imply that disturbance to maternal care or to the delicate mother-pup relationship may explain differential patterns of behavior in the offspring rather than direct effects of prenatal exposure to a toxicant.

Aluminium (Al) is a ubiquitous element used extensively in our modern environment. In recent years, it has been demonstrated that Al is involved in a number of human pathological conditions. Unlike other heavy metals e.g lead and methylmercury, the development toxicity of Al has been little considered. In previous studies (Clayton, *et al.*, 1992; Rankin and Manning, 1993), we have reported several developmental effects of prenatal exposure to Al including body weight changes, transient delays in several tests for sensory-motor development and alterations to ultrasonic calling. Moreover, the treatment received by a pup's foster mother was found to influence several of these measures. Alterations to the quantity or quality of maternal behavior following exposure to Al during pregnancy was suggested as a possible explanation for the maternal treatment effect on pup weight. For this reason, we have undertaken to characterise in two inbred strains of mice (CBA and C57BL/6J), which are known to differ in several behavioral and neurochemical parameters (Sedowofia, *et*

al., 1989), any changes to the pattern of maternal behavior of control and Al-treated mothers. A comprehensive array of behaviors was considered to enable assessment of the effects of Al exposure not only on the overall pattern of maternal behavior but also on individual components of it.

Only one previous study in rats has included simultaneous recording of maternal behavior after prenatal exposure to Al (Bernuzzi, *et al.*, 1986). These authors reported no significant differences between treatment groups in nest-building, retrieval of pups to the nest or time spent with the young following exposure to $AlCl_3$ (160 or 200mg Al/kg body weight/day) in the dam's diet from day 8 to 20 of gestation.

METHODS

Animals and Treatment

All mice were maintained in an air-conditioned room at 21±1°C and 60±10% relative humidity, with a 12 hour light cycle. Pellet food (enriched standard diet purchased from Piccioni I-2500 Brescia, Italy) and tap water were freely available. Females were mated with males from the same strain.

Pregnant females were randomly divided into control and treated groups. Treated females were exposed to $Al_2(SO_4)_3$ at a dose of 200mg/kg body weight during gestation days 10 to 13. The dose range and the schedule of exposure were selected on the basis of previous literature and experiments (Ondreika, *et al.*, 1966; Clayton, *et al.*, 1992). Maternal weights were recored during gestation and the amount of food and water ingested were determined during this period.

Maternal Behavior

On the day of birth (postnatal day 0, Pd0), all pups were weighed and each litter was culled to four. Pups were intrastrain cross-fostered using a balanced split-litter total cross-foster procedure (Chariotti, *et al.*, 1987; Sedowofia, *et al.*, 1989), with other litters born within the same 12 hour period. Each mother received two control and two treated pups from other litters and none of which were her own. Following cross-fostering on Pd1, each mother was housed in a clear plastic cage, to facilitate maternal behavior observation. The cage contained shavings and 1g of nesting material consisting of strips of paper. The cages were not cleaned until maternal behavior observations were completed. The procedure employed for maternal behavior observation was based on that described by Laviola and colleagues (1990). 10 minute recording sessions occurred

twice daily (during the initial and final hours of the dark phase) under red light and were randomised so that the treatment groups were equally represented at different times. Dams were placed in the experimental room 30 minutes prior to commencement of recording and remained there until the end of the day. The cage lids were not replaced by a perspex one for two reasons. Firstly, pilot studies showed that the changeover disturbed the mother and increased exploration of the new cage lid. Secondly, leaving the standard lid, made it possible to include the recording of *eating and drinking* behaviour. Although these behaviors are clearly not part of the maternal behavior repertoire, their recording may be important in assessing any influence of Al exposure on milk production. Also recording was continued to include a later age, day 18 postpartum, to investigate whether exposure to Al during gestation prolonged the expression of any behaviors.

Observations were made on postnatal days 2, 3, 5, 7, 10, 14 and 18 but not on Pd1 due to possible neophobic reactions to the new cage and cross-fostering. These time periods are thought to correspond with major developmental and neurochemical changes within the infant mouse (Sedowofia, *et al.*, 1989). Behavior was recorded on a keyboard linked to a computer which recorded the frequency and duration of each key press as mutually exclusive and independent events (Deag, 1983a). The information was stored on computer discs for subsequent analysis (Deag, 1983b). The following elements of maternal behavior were recorded:

Pup-directed behaviors

Nursing - The mother adopts the classic lactation position, arching her body over the pups. Nursing was recorded even when the mother was seen in the nest grooming herself but having at least one pup attached to a nipple, a condition which was common during the later ages.

Licking - Scored when the mother is solely actively engaged in licking any part of a pup's body.

Nest-building - This is recorded when the mother is in the nest and pulling shavings into it, is carrying material to the nest or is using her forepaws to push material towards it.

Retrieving - The mother returns a pup which had wandered from the nest site. In a laboratory situation such as this, retrieval of a pup is rarely seen.

If the mother was *nursing* but also engaged in one of the other pup-directed activities, the former was scored to record any actual nourishment received by the pups in preference to other maternal activities occuring at the same time, in order to estimate if the pups were being affected by postnatal events.

Non pup-directed activities

Active - The mother is out of the nest and is moving around the cage.

Rearing - The mother is on her hind legs leaning against the cage walls.

Grooming - The mother is licking any part of her own body.

Still - The mother is lying still with no pups attached either beside the nest or at the other end of the cage.

Eating and Drinking - The mother is eating from the food hopper or drinking from the water bottle.

At the end of the 10 minutes recording period the quality of the nest was scored on a scale of 0 to 4; a score of 0 was given when there was no discernible nest and the pups were scattered, and a nest was judged to have a score of 4 when it was well constructed and the pups were not obviously visible.

STATISTICAL ANALYSIS

Each of the control and treated maternal behaviors were compared using Mann-Whitney U-tests on each test day. Maternal weight, and food and water intake were analysed by Student's *t*-tests. The nest scores were analysed using analysis of variance (Dixon, 1988).

RESULTS

Level of aluminium exposure

Pregnant females, with an average weight of 25g, injected i.p with $Al_2(SO_4)_3$ received a total of 1.64mg over the four days of treatment.

Maternal weight

Within the CBA strain there were 14 control and 15 treated pregnant females and 9 control and 12 treated females in the C57 strain. The greater number of CBA females resulted from the experiment being set up twice. For both strains weight gain during the treatment period (Gd10 to 13) was slight but increased greatly after its termination. Control and treated females gained weight at a comparable rate in both strains, although CBA control females weighed about 5% more than treated females by day 18 of gestation.

As with prenatal weights, the weights recorded for control and treated dams from day 3 postpartum to weaning were not significantly different either between treatment groups or strains.

Food and water intake

There were no differences in the amount of food and water ingested by control and treated CBA females during gestation days 10-18. Food intake was similar for control and treated C57 females, however water consumption was slighlty reduced for treated C57 females (control n=9 x=77.4($\pm$2.8)g, treated n=12 x=68.2($\pm$1.8)g, $t(14)=2.76$ $p<0.02$).

Maternal Behavior

For the purposes of analysis, maternal behavior scores for the two daily sessions were combined in the absence of any reliable differences between the two test sessions. Figures 5.1 and 5.3 summarize the most important differences in maternal activities for control and treated mothers over the 7 test days for CBA (7 control and 6 treated) and C57 (6 control and 8 treated) females. The graphs represent the mean duration and frequency of bouts of the pup-directed maternal behaviors (nursing, licking) and the non pup-directed activities (combined eating and drinking). Although individual comparisons of each behavior between treatment groups were analysed using nonparametric tests, the means are presented rather than the medians due to the frequent occurrence of zero scores. Pup retrieval was not observed on any test day.

In accordance with the Laviola *et al.*, (1990) study and others (Ewart and Cutler, 1979; Chapman and Cutler, 1983) maternal care was, in general, more pronounced during the first few days after birth in both treatment groups and for both strains, and subsequently declined as the pups matured, being replaced by non pup-directed activities. Although there were few significant differences between control and treated mothers for any of the behaviors measured, the graphs reveal clear and consistent trends which are described below.

Nursing - The frequency of bouts of *nursing* was similar for control and treated CBA females during the test period. However, control CBA dam's spent an increased amount of time involved in *nursing* their offspring especially during the latter test days. The duration of this behavior during these days was less than during the initial postpartum days.

From days 3 to 10 postpartum Al-exposed C57 mothers showed an increased number of bouts of *nursing* which were of longer duration than control mothers but only reached marginal significance on Pd5.

FIGURE 1 HERE

Licking - Low levels of *licking* behavior were recorded for both control and treated mothers in both strains throughout the test period. On Pd10 and 14 CBA treated dam's exhibited an increased number of bouts of *licking* of longer duration than control CBA females which were significantly different on Pd14 (frequency $U=56.0$ $p<0.03$; duration $U=56.0$ $p<0.03$).

The frequency and duration of *licking* behavior on all test days except Pd18 were slightly greater for treated C57 mothers than controls. For both control and treated mothers *licking* behavior increased as a function of age and peaked on Pd10, although this difference was not significant.

FIGURE 2 HERE

Nest-building - Very low levels of this behavior were exhibited by either control or treated females from either strain.

As described earlier, non pup-directed behaviors were rare during the first postnatal week but became more predominant thereafter.

Active - The frequency and duration of bouts of *active* behavior increased from Pd7 onwards for both control and Al-exposed CBA females. On Pd14 treated CBA females showed an increased number of these bouts which were of longer duration but did not reach significance.

C57 mothers did not show such a clear pattern of *active* behavior, although the frequency and duration of this behavior was greater from Pd10 onwards. As with CBA Al-exposed females, treated C57 females showed a trend towards an increase in the number and duration of bouts of *active* behavior compared to controls, although this was not significant.

Still - Mothers of both strains and both groups showed an increased frequency and duration of bouts of *still* behavior during the second postnatal week. Control CBA females tended towards a greater amount of this behavior during Pd7, 10 and 14 although this difference was not significant.

The frequency of *still* behavior was similar for C57 control and treated mothers, although control C57 females tended towards spending more time involved in this behavior.

Groom - The frequency and duration of bouts of *grooming* behavior increased as a function of age for CBA control mothers. However, Al-exposed CBA females showed a significant increase in the frequency ($U=42.0$ $p<0.03$) and duration ($U=39.5$ $p<0.02$) of *grooming* behavior during Pd5.

The frequency and duration of *grooming* behavior was similar throughout the test period for control and treated C57 females except on Pd5 when control C57 mothers spent more time involved in *grooming* ($U=133.0$ $p< 0.04$).

Rear - The frequency and duration of *rearing* behavior increased from Pd3 onwards for CBA mothers and both control and treated groups showed similar levels of this behavior. However, on Pd14 Al-exposed CBA mothers showed a significantly greater number of bouts of *rearing* ($U=43.0$ $p<0.03$) and of longer duration ($U=41.0$ $p<0.02$) than controls.

The frequency of *rearing* for both C57 control and treated groups declined between Pd2 to 7 and then increased. Treated C57 females showed a greater number of bouts of *rearing* which were of longer duration than control mothers but did not reach significance.

Eating + Drinking - Control and treated CBA mothers showed similar frequency and duration of bouts of *eating* and *drinking* which increased slightly from Pd3 to 18. However, on Pd10 treated CBA females exhibited an increased frequency of bouts of *eating and drinking* which were of longer duration, but this difference was only marginally significant.

The frequency of food and water ingestion increased from Pd2 to 7 and then declined for C57 control females but steadily increased from Pd5 for Al-treated C57 mothers. Control C57 females ingested more food and water during Pd5-10 than treated females, although this difference did not reach significance.

FIGURE 3 HERE

Nest quality score

The total score of nest quality over the 7 test days showed that both control and treated CBA and C57 mothers built nests to a similar standard (CBA control x=39.7(±1.3), treated x=40.5(±1.4); C57 control x=41.8(±1.0), treated x=40.3(±1.1)).

DISCUSSION

The results of these observations, which examined the effects of exposure to Al during a limited period of gestation on a number of maternal activities, revealed only slight differences between control and Al-treated mothers and then only on certain days. The pattern of some components of maternal behavior depended both on prior treatment and on the strain of the mother whilst others were only treatment dependent. Al-exposed CBA mothers spent less time involved in *nursing* their offspring whilst C57 treated dams showed enhanced *nursing* behavior compared to controls. However in the case of *licking*, the Al-treated mothers from both strains spent more time involved in this behavior. The low level of *licking* behavior may have resulted from the preferential recording of *nursing* behavior if both behaviors occurred simultaneously. CBA treated mothers tended towards increased involvement in non pup-directed activities.

Hence, only slight differences in certain elements of maternal behavior were found which are unlikely to reflect alterations in the ability of the mother to respond maternally towards her young. Exposure to Al did not affect the mechanisms responsible for the expression and organisation of maternal behavior, which is in accordance with Bernuzzi, *et al.*, (1986). Presumably the level of pup stimulation was equal for control and treated dams, as each litter consisted of two control and two Al-treated pups. There is no evidence to suggest a decrease in the ability of the pups to elicit adequate maternal responses.

The impairment in weight gain by CBA pups fostered to treated mothers described in Clayton, *et al.*, (1992), can not be explained by deficits in availability of nourishment, as treated CBA mothers showed similar levels of *nursing, eating* and *drinking* behavior as controls. However, although mothers were seen in the *nursing* position, lying with an arched back over the pups, the possiblity exists that the pups were not always actively involved in suckling, or that the milk is not of the same quality.

There was only a slight difference in the amount of food and water ingested by control and treated CBA and C57 mothers during gestation. Thus the body weight differences did not result from undernourishment of the treated mothers which has been shown to result in less efficient retrieval of young and in a reduction in *licking* (Smart and Preece, 1973).

Despite the lack of significant effects of exposure to Al during gestation on maternal behavior, Al had toxic effects on other maternal characteristics. In both this experiment and the one described in Clayton *et al.*, (1992), pregnant CBA females exposed to Al via i.p. injection gained less weight during gestation than that of control females, although this difference was not significant here. However, maternal weights during lactation did not differ between control and treated mothers, suggesting that the influence of any decrease in body weight which occurred prenatally was overcome.

The unlikely contribution of differences in maternal care points to a direct effect of Al on the physiology of the mother. Exposure to Al during pregnancy has been shown to reduce milk consumption of rabbit offspring in a dose-dependent manner (Yokel, 1984; Yokel, 1985). This is the case for both treated and control pups suckling from Al-exposed lactating does. Thus, the maternal treatment effect is not likely to be mediated via changes in the ability of the Al-exposed mothers to care for their young but effects of Al on milk production may be of greater relevance. Moreover, the nutritional value of the milk produced by Al-exposed mothers may be inferior to that produced by control mothers.

Finally, Al may exert its effects directly on the pup. A decrease in milk yield may be accompanied by a decline in attempts made by the offspring to suckle from treated mothers. As milk output and Al content were not assessed during this experiment these possibilities remain speculative.

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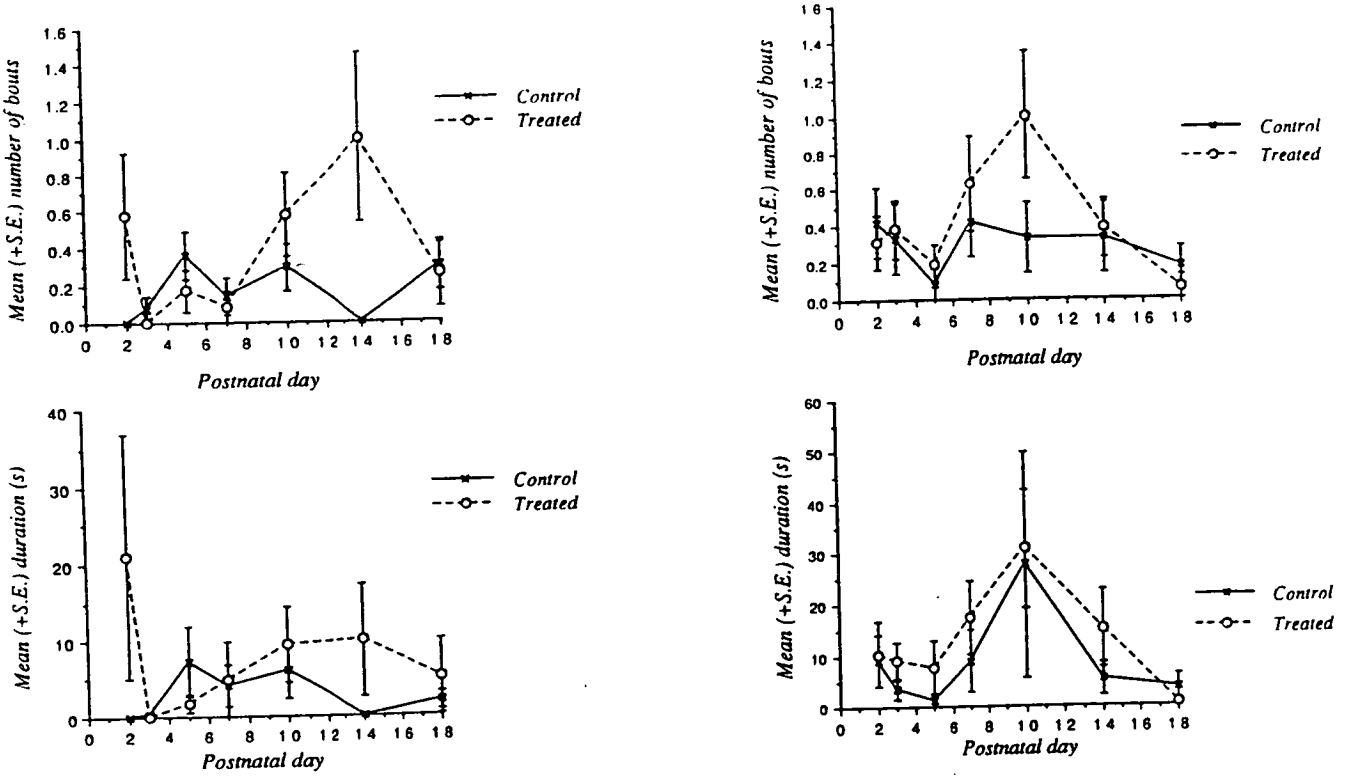


Fig. 1: Effect of gestational exposure to aluminium sulphate (200mg/kg, Gd10-13) on the frequency and duration of bouts of *nursing* behaviour for (a) CBA and (b) C57BL/6J dams.

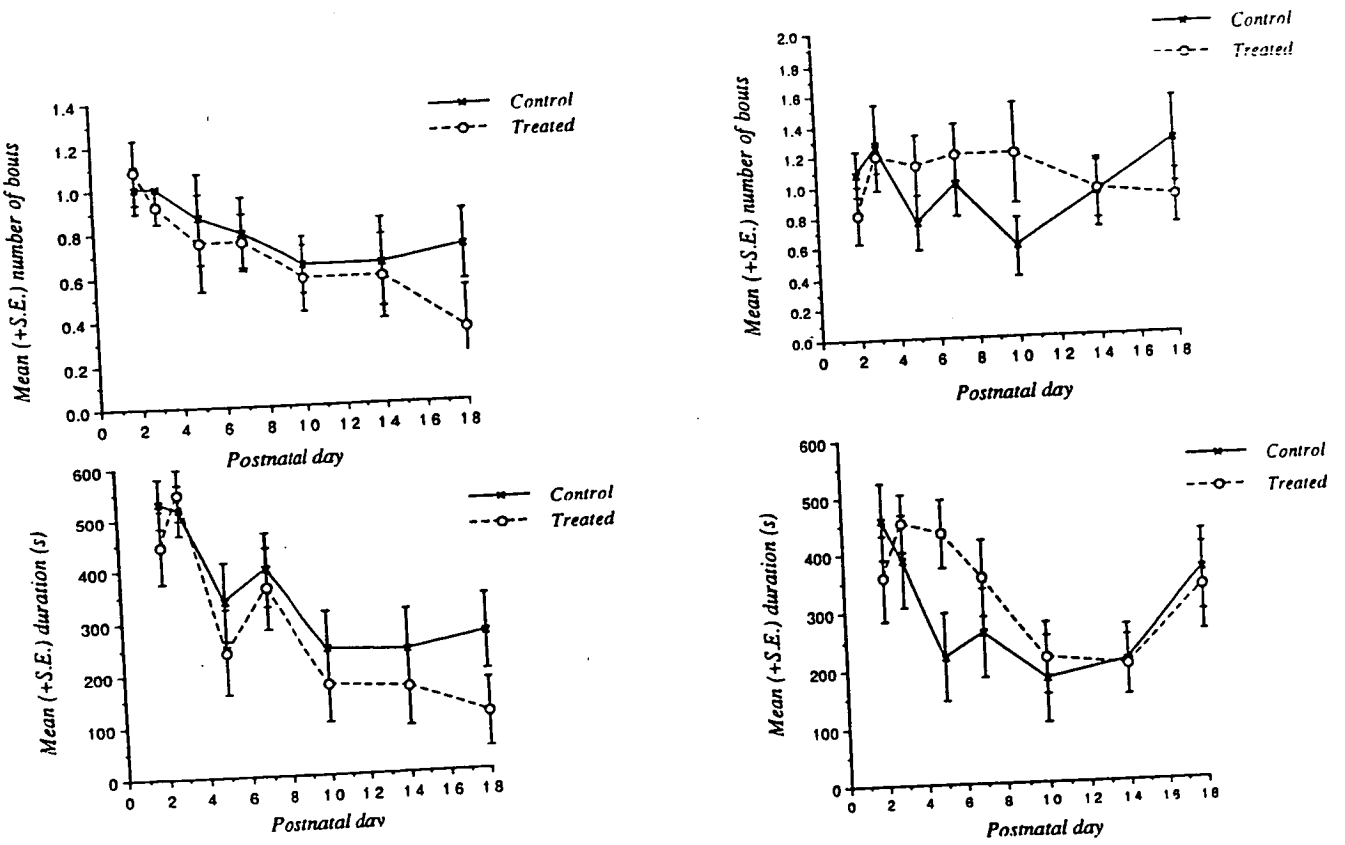
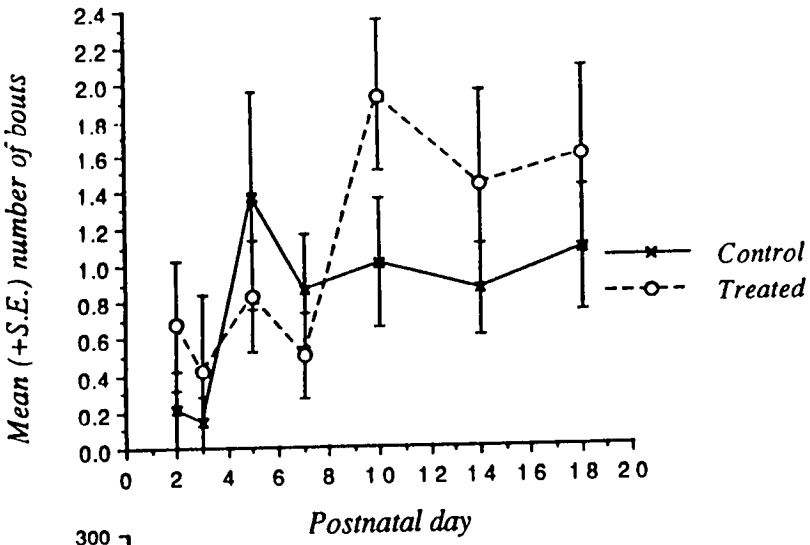
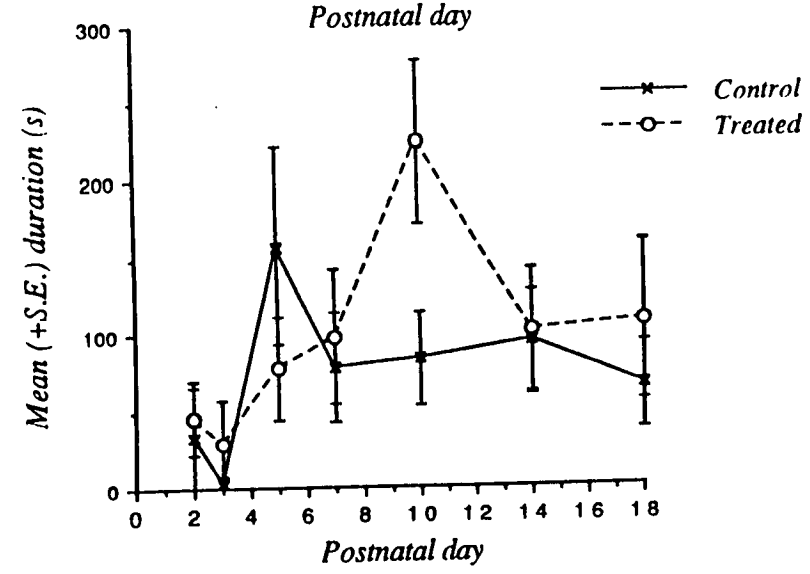
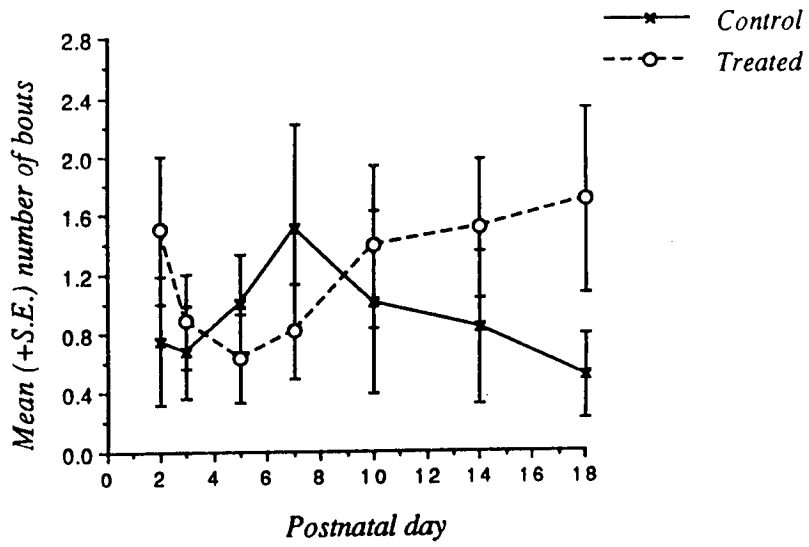


Fig. 2: Effect of gestational exposure to aluminium sulphate (200mg/kg, Gd10-13) on the frequency and duration of bouts of *licking* behaviour for (a) CBA and (b) C57BL/6J dams.







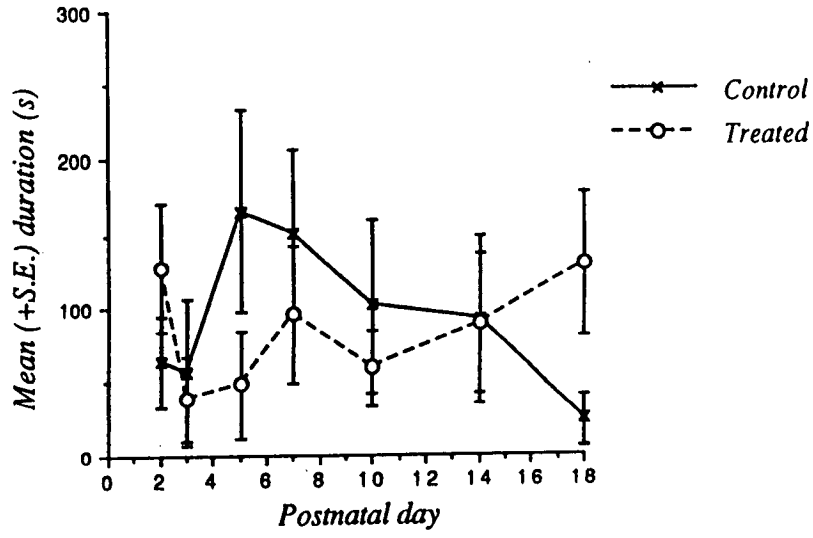


Fig. 3: Effect of gestational exposure to aluminium sulphate (200mg/kg, Gd10-13) on the frequency and duration of bouts of *eating and drinking* for (a) CBA and (b) C57BL/6J dams.

APPENDIX F

ANOVA table of CBA pup activity on Pd15 and 18 following exposure to aluminium sulphate (200mg/kg, Gd10-13).

SOURCE	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARES	F VALUE	TAIL PROB.
MEAN	163358.23171	1	163358.23171	442.65	0.0000
TREAT	1170.73171	1	1170.73171	3.17	0.0909
FMAT	52.24573	1	52.24573	0.14	0.7109
SEX	545.33415	1	545.33415	1.48	0.2390
TF	8.70915	1	8.70915	0.02	0.8795
TS	49.20000	1	49.20000	0.13	0.7190
FS	76.87500	1	76.87500	0.21	0.6533
TFS	159.19207	1	159.19207	0.43	0.5192
ERROR	7011.80000	19	369.04211		
DAY	418.54167	1	418.54167	2.42	0.1364
DT	22.80996	1	22.80996	0.13	0.7206
DF	123.02520	1	123.02520	0.71	0.4096
DS	8.37581	1	8.37581	0.05	0.8282
DTF	1120.54959	1	1120.54959	6.47	0.0198
DTS	180.74167	1	180.74167	1.04	0.3196
DFS	19.53252	1	19.53252	0.11	0.7406
DTFS	9.83740	1	9.83740	0.06	0.8141
ERROR	3288.13333	19	173.05965		

C57BL/6J FEMALES

SOURCE	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARES	F VALUE	TAIL PROB.
MEAN	196692.09069	1	196692.09069	418.88	0.0000
TREAT	46.69563	1	46.69563	0.10	0.7539
FMAT	42.27304	1	42.27304	0.09	0.7655
SEX	177.23680	1	177.23680	0.38	0.5419
TF	10.56909	1	10.56909	0.02	0.8814
TS	2645.21237	1	2645.21237	5.63	0.0218
FS	315.97749	1	315.97749	0.67	0.4162
TFS	149.63409	1	149.63409	0.32	0.5751
ERROR	22069.40813	47	469.56188		
DAY	16419.61321	1	16419.61321	50.58	0.0000
DT	31.26372	1	31.26372	0.10	0.7577
DF	278.26372	1	278.66254	0.86	0.3589
DS	193.32081	1	193.32081	0.60	0.4442
DTF	167.43348	1	167.43348	0.52	0.4762
DTS	2170.23695	1	2170.23695	6.69	0.0129
DFS	535.23289	1	535.23289	1.65	0.2054
DTFS	51.89452	1	51.89452	0.16	0.6911
ERROR	15257.16131	47	324.62045		

ANOVA table showing the effects of a scopolamine challenge on CBA pup activity at weaning. The sex variable was not included in this analysis as some of the sample sizes would have been one.

SOURCE	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARES	F VALUE	TAIL PROB.
MEAN	419469.08741	1	419469.08741	507.19	0.0000
TREAT	0.15306	1	0.15306	0.44	0.5112
FMAT	2174.45984	1	2174.45984	2.63	0.1154
CHALL	53684.51265	1	53684.51265	64.91	0.0000
TF	325.82294	1	325.82294	0.39	0.5350
TC	4289.61712	1	4289.61712	5.19	0.0301
FC	1311.10761	1	1311.10761	1.59	0.2177
TFC	394.85595	1	394.85595	0.48	0.4949
ERROR	24811.43667	1	827.04789		
BK	33170.16097	4	8292.54024	23.33	0.0000
BT	1316.31825	4	329.07956	0.93	0.4443
BF	932.22155	4	233.05539	0.66	0.6083
BC	4689.25223	4	1172.31306	3.30	0.0168
BTF	2367.55165	4	591.88791	1.67	0.1694
BTC	821.69534	4	205.42383	0.58	0.6614
BFC	1466.23010	4	366.55752	1.03	0.3901
BTFC	1454.24291	4	363.56073	1.02	0.3942
ERROR	42651.18000	120	355.42650		

C57BL/6J

SOURCE	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARES	F VALUE	TAIL PROB.
MEAN	945159.57818	1	945159.57818	727.99	0.0000
TREAT	3.34586	1	3.34586	0.00	0.9598
FMAT	879.83520	1	879.83520	0.68	0.4154
SEX	234.80893	1	234.80893	0.18	0.6730
CHALL	22149.17818	1	22149.17818	17.06	0.0002
TF	1617.23924	1	1617.23924	1.25	0.2712
TS	11610.80691	1	11610.80691	8.94	0.0048
FS	1257.25091	1	1257.25091	0.97	0.3312
TC	17.98828	1	17.98828	0.01	0.9069
FC	1079.21028	1	1079.21028	0.83	0.3675
SC	262.26213	1	262.26213	0.20	0.6556
TFS	808.21769	1	808.21769	0.62	0.4349
TFC	407.69109	1	470.69109	0.36	0.5506
TSC	2113.89648	1	2113.89648	1.63	0.2095
FSC	19.43722	1	19.43722	0.01	0.9032
TFSC	4510.01253	1	4510.01253	3.47	0.0699
ERROR	50634.57333	39	1298.32239		
BK	44971.67962	4	11242.91990	39.23	0.0000
BT	732.69309	4	183.17327	0.64	0.5954
BF	1800.28456	4	450.07114	1.57	0.1990
BS	1116.40061	4	279.10015	0.97	0.4092
BC	7298.55773	4	1824.63943	6.37	0.0004
BTF	129.01744	4	32.25436	0.11	0.9556
BTS	3231.69780	4	807.92445	2.82	0.0405
BFS	1107.77232	4	276.94308	0.97	0.4128
BTC	460.90813	4	115.22703	0.40	0.7571

BFC	3044.43585	4	761.10896	2.66	0.0500
BSC	1494.48725	4	373.62181	1.30	0.2762
BTFS	713.65403	4	178.41351	0.62	0.6060
BTFC	1060.68568	4	265.17142	0.93	0.4329
BTSC	1287.20756	4	321.80189	1.12	0.3434
BFSC	1652.46817	4	413.11704	1.44	0.2334
BTFSC	384.92316	4	96.23079	0.34	0.8048
ERROR	44706.06000	156	286.57731		

MALES

SOURCE	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARES	F VALUE	TAIL PROB.
MEAN	515024.79093	1	515024.79093	394.10	0.0000
TREAT	6311.22427	1	6311.22427	4.83	0.0388
FMAT	2385.32730	1	2385.32730	1.83	0.1904
CHALL	15317.86972	1	15317.86972	11.72	0.0024
TF	78.13336	1	78.13336	0.06	0.8091
TC	979.80912	1	979.80912	0.75	0.3959
FC	780.92730	1	780.92730	0.60	0.4477
TFC	4440.76063	22	4440.76063	3.40	0.0788
ERROR					
BK	31502.03470	4	7875.50867	27.50	0.0000
BT	3359.30591	4	839.82648	2.93	0.0396
BF	1978.87712	4	494.71928	1.73	0.1697
BC	6894.72359	4	1723.68090	6.02	0.0011
BTF	522.69985	4	130.67496	0.46	0.7146
BTC	1341.28672	4	335.32168	1.17	0.3277
BFC	4096.28722	4	1024.07181	3.58	0.0183
BTFC	542.33520	4	135.58380	0.47	0.7027
ERROR	25205.26000	88	286.42341		

FEMALES

SOURCE	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARES	F VALUE	TAIL PROB.
MEAN	438835.11566	1	438835.11566	340.90	0.0000
TREAT	5403.75808	1	5403.75808	4.20	0.0562
FMAT	15.11566	1	15.11566	0.01	0.9150
CHALL	7916.00051	1	7916.00051	6.15	0.0239
TF	2120.40455	1	2120.40455	1.65	0.2166
TC	1134.84899	1	1134.84899	0.88	0.3609
FC	364.04091	1	364.04091	0.28	0.6018
TFC	930.02475	1	930.02475	0.72	0.4071
ERROR	21883.95000	17	1287.29118		
BK	16277.64444	4	4069.41111	14.19	0.0000
BT	880.50707	4	220.12677	0.77	0.5116
BF	1034.14949	4	258.53737	0.90	0.4430
BC	2397.96162	4	599.49040	2.09	0.1167
BTF	340.24444	4	85.06111	0.30	0.8175
BTC	500.27474	4	125.06869	0.44	0.7181
BFC	950.18384	4	237.54596	0.83	0.4794
BTFC	867.17980	4	216.79495	0.76	0.5180
ERROR	19500.80000	68	286.77647		

APPENDIX G

ANOVA table of C57BL/6J pup body weights following prenatal exposure to oral aluminium sulphate (750mg/L, Gd10-16).

SOURCE	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARES	F VALUE	TAIL PROB.
MEAN	12169.18231	1	12169.18231	4903.24	0.0000
TREAT	0.55307	1	0.55307	0.22	0.6397
FMAT	14.10039	1	14.10039	5.68	0.0225
SEX	0.83423	1	0.83423	0.34	0.5657
TF	0.19022	1	0.19022	0.08	0.7835
TS	0.50868	1	0.50868	0.20	0.6535
FS	0.82955	1	0.82955	0.33	0.5668
TFS	0.02248	1	0.02248	0.01	0.9247
ERROR	89.34712	36	2.48186		
DAY	2036.30605	6	339.38434	2410.53	0.0000
DT	0.09402	6	0.01567	0.11	0.8609
DF	1.51312	6	0.25219	1.79	0.1805
DS	1.18039	6	0.19673	1.40	0.2539
DTF	0.32886	6	0.05481	0.39	0.6423
DTS	0.72906	6	0.12151	0.86	0.4096
DFS	0.74254	6	0.12376	0.88	0.4037
DTFS	0.25897	6	0.04316	0.31	0.6981
ERROR	30.41115	216	0.14079		

C57BL/6J 1250mg/L

SOURCE	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARES	F VALUE	TAIL PROB.
MEAN	9649.87736	1	9649.87736	4353.66	0.0000
TREAT	0.00257	1	0.00257	0.00	0.9730
FMAT	21.51843	1	21.51843	9.71	0.0038
SEX	0.00990	1	0.00990	0.00	0.9471
TF	0.00704	1	0.00704	0.00	0.9554
TS	2.56079	1	2.56079	1.16	0.2902
FS	0.25568	1	0.25568	0.12	0.7363
TFS	1.41025	1	1.41025	0.64	0.4308
ERROR	73.14448	33	2.21650		
DAY	1799.44635	6	299.90773	1752.76	0.0000
DT	0.49208	6	0.08201	0.48	0.6717
DF	1.05527	6	0.17588	1.03	0.3773
DS	0.28553	6	0.04759	0.28	0.8142
DTF	0.72122	6	0.12020	0.70	0.5344
DTS	0.73541	6	0.12257	0.72	0.5268
DFS	0.13918	6	0.02320	0.14	0.9194
DTFS	0.37749	6	0.06292	0.37	0.7490
ERROR	33.87892	198	0.17111		

ANOVA table of C57BL/6J adult female body weights following prenatal exposure to oral aluminium sulphate (750mg/L, Gd10-16).

SOURCE	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARES	F VALUE	TAIL PROB.
MEAN	99826.39858	1	99826.39858	8705.07	0.0000
TREAT	0.14746	1	0.14746	0.01	0.9114
FMAT	18.62287	1	18.62287	1.62	0.2249
TF	3.36288	1	3.36288	0.29	0.5973
ERROR	149.07901	13	11.46762		
WEEK	1577.75402	12	131.47950	469.37	0.0000
WT	2.56395	12	0.21366	0.76	0.5738
WF	8.62631	12	0.71886	2.57	0.0383
WTF	3.61746	12	0.30145	1.08	0.3810
ERROR	43.69869	156	0.28012		

Median (+ lower and upper interquartile ranges) number of ultrasonic calls produced following exposure to oral aluminium sulphate .

CBA 1000mg/L

Day	Cc	Ct	Tt	Tc
3	61.0 (41.5, 203.5)	59.0 (15.0, 217.0)	24.0 (17.0, 103.0)	47.5 (12.2, 100.2)
4	110.0 (35.7, 198.7)	143.0 (22.0, 239.0)	70.0 (17.0, 151.0)	91.0 (31.0, 134.0)
5	250.5 (123.2,277.2)	55.0 (21.5, 219.0)	66.0 (23.0, 166.0)	54.5 (31.2, 151.0)
6	114.5 (66.0, 159.2)	62.0 (27.0, 180.0)	67.0 (14.0, 97.0)	69.0 (50.0, 166.2)
9	49.0 (9.2, 129.7)	46.0 (22.5, 132.0)	27.0 (4.0, 64.0)	25.5 (11.5, 49.5)
12	8.5 (6.2, 116.7)	23.0 (8.5, 111.5)	11.0 (4.0, 74.0)	33.0 (11.7, 76.5)

C57BL/6J

Day	Cc	Ct	Tt	Tc
3	34.5 (10.5, 91.7)	7.0 (3.5, 35.0)	21.0 (6.0, 33.0)	14.0 (3.0, 23.0)
4	21.5 (10.8, 35.0)	13.0 (5.0, 18.5)	8.0 (3.5, 53.5)	11.0 (5.0, 16.5)
5	9.0 (5.3, 22.0)	5.0 (3.5, 13.0)	10.0 (5.5, 21.5)	9.0 (4.0, 11.5)
6	5.5 (3.0, 30.0)	14.0 (7.5, 21.0)	6.0 (3.5, 19.0)	11.0 (3.5, 15.0)
9	1.5 (1.0, 4.8)	15.0 (6.0, 33.0)	4.0 (3.0, 13.5)	3.0 (2.0, 3.5)
12	1.0 (0.8, 4.0)	1.0 (0.5, 21.0)	2.0 (1.0, 4.0)	1.0 (0.5, 3.0)

CBA 1250mg/L

Day	Cc	Ct	Tt	Tc
3	23.5 (6.0, 88.8)	43.0 (11.0, 69.0)	26.0 (14.5, 61.0)	12.0 (0.0, 55.3)
4	125.0 (67.3, 163.3)	119.0 (89.0, 254.0)	96.5 (62.3, 181.3)	72.0 (19.0, 131.2)
5	117.0 (25.5, 250.8)	117.0 (32.0, 223.0)	97.0 (48.5, 197.5)	46.0 (11.5, 116.7)
6	120.0 (63.3, 255.7)	101.0 (32.0, 223.0)	75.5 (54.5, 139.5)	92.0 (56.7, 174.3)
9	74.0 (54.0, 157.8)	46.0 (17.0, 126.0)	50.0 (31.8, 96.2)	66.5 (47.5, 99.2)
12	50.5 (24.2, 75.8)	31.0 (6.0, 119.0)	17.0 (0.0, 58.5)	48.5 (21.5, 123.5)

C57BL/6J

Day	Cc	Ct	Tt	Tc
3	6.0 (2.0, 15.5)	8.0 (0.0, 25.3)	5.0 (2.3, 13.8)	7.5 (2.5, 25.8)

4	4.0 (2.0, 9.5)	5.5 (1.5, 15.0)	10.0 (1.0, 20.0)	5.5 (0.5, 18.0)
5	10.0 (4.0, 18.0)	8.5 (5.5, 17.8)	12.0 (6.3, 20.8)	14.5 (10.5, 27.5)
6	9.0 (3.0, 36.0)	8.0 (4.5, 22.3)	5.5 (0.8, 1.3)	14.5 (7.0, 37.0)
9	0.0 (0.0, 1.0)	0.0 (0.0, 0.0)	0.5 (0.0, 4.0)	0.0 (0.0, 3.8)
12	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)	0.0 (0.0, 0.5)	0.0 (0.0, 0.0)

ANOVA of pup activity at weaning following prenatal exposure to oral aluminium sulphate.

CBA 1000mg/L

SOURCE	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARES	F VALUE	TAIL PROB.
MEAN	121661.07429	1	124661.07429	452.06	0.0000
TREAT	336.80048	1	336.80048	1.25	0.2728
FMAT	1501.98857	1	1501.98857	5.58	0.0253
SEX	31.32964	1	31.32964	0.12	0.7355
TF	123.37190	1	123.37190	0.46	0.5039
TS	161.02012	1	161.02012	0.60	0.4457
FS	359.18679	1	359.18679	1.33	0.2577
TFS	10.22012	1	10.22012	0.04	0.8469
ERROR	7535.54000	28	269.12643		
MIN	144.93137	4	36.23284	0.67	0.5979
MT	196.09804	4	49.02451	0.91	0.4530
MF	178.73137	4	44.68284	0.83	0.4991
MS	575.65220	4	143.91305	2.68	0.0407
MTF	331.13375	4	82.78344	1.54	0.2008
MTS	95.09268	4	23.77317	0.44	0.7603
MFS	364.58077	4	91.14519	1.70	0.1624
MTFS	267.35696	4	66.83924	1.24	0.2980
ERROR	6021.00667	112	53.76095		

CBA 1250mg/L

SOURCE	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARES	F VALUE	TAIL PROB.
MEAN	203336.68848	1	203336.68848	1023.19	0.0000
TREAT	0.01259	1	0.01259	0.00	0.9937
FMAT	111.29922	1	111.29922	0.56	0.4569
SEX	23.35319	1	23.35319	0.12	0.7328
TF	27.95127	1	27.95127	0.14	0.7088
TS	836.79316	1	836.79316	4.21	0.0441
FS	261.76546	1	261.76546	1.32	0.2552
TFS	41.26080	1	41.26080	0.21	0.6501
ERROR	13314.74187	67	198.72749		
MIN	1131.85850	4	282.96463	3.85	0.0055
MT	144.32267	4	36.08067	0.49	0.7327
MF	412.52710	4	103.13178	1.40	0.2359
MS	720.74733	4	180.18683	2.45	0.0500
MTF	563.76726	4	140.94182	1.92	0.1121
MTS	276.82765	4	69.20691	0.94	0.4374
MFS	262.84085	4	65.71021	0.89	0.4642
MTFS	214.45363	4	53.61341	0.73	0.5660
ERROR	19718.35485	268	73.57595		

EDGEON DURATION

Day	Cc	Ct	Tt	Tc
15	0.0 (0.0, 1.7)	5.2 (0.5, 21.7)	5.2 (2.5, 14.3)	6.0 (0.8, 12.6)
18	14.8 (8.9, 17.7)	22.8 (7.1, 33.2)	16.8 (9.0, 28.6)	20.3 (15.8, 27.6)

FREQUENCY

Day	Cc	Ct	Tt	Tc
15	0.0 (0.0, 1.3)	4.0 (0.5, 10.5)	4.0 (1.0, 8.5)	4.5 (0.8, 10.5)
18	14.0 (9.0, 16.0)	16.0 (7.3, 19.0)	15.5 (8.3, 19.3)	16.0 (11.0, 20.5)

RIGHTING DURATION

Day	Cc	Ct	Tt	Tc
3	41.1 (19.4, 62.2)	16.7 (0.0, 41.6)	4.3 (0.0, 23.9)	0.0 (0.0, 43.2)
6	2.4 (0.0, 41.3)	0.0 (0.0, 0.0)	0.0 (0.0, 10.7)	0.0 (0.0, 8.3)

ACTIVITY

SOURCE	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARES	F VALUE	TAIL PROB.
MEAN	215631.39358	1	215631.39358	358.96	0.0000
TREAT	0.29433	1	0.29433	0.00	0.9825
FMAT	2806.79634	1	2806.79634	4.67	0.0370
TF	403.50306	1	403.50306	0.67	0.4176
ERROR	22827.32605	38			
DAY	190859.17177	3	63619.72392	125.99	0.0000
DT	1759.14507	3	586.38169	1.16	0.3169
DF	3758.68312	3	1252.89437	2.48	0.0886
DTF	977.33199	3	325.77733	0.65	0.5195
ERROR	57566.54563	114			

ANOVA table of the effects of a scopolamine challenge on the number of trials required by C57BL/6J males to enter all 8 arms in a radial maze.
Foster mother treatment was not taken into consideration.

NUMBER OF TRAILS

SOURCE	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARES	F VALUE	TAIL PROB.
MEAN	3957.02703	1	3957.02703	384.52	0.0000
TREAT	21.89189	1	21.89189	2.13	0.1727
CHALL	81.82703	1	81.82703	7.95	0.0167
TC	0.09730	1	0.09730	0.01	0.9243
ERROR	113.20000	11	10.29091		
DAY	8.22342	2	4.11171	1.09	0.3429
DT	53.84505	2	26.92252	7.14	0.0084
DC	6.10450	2	3.05225	0.81	0.4334
DTC	42.64505	2	21.32252	5.66	0.0179
ERROR	82.93333	22	3.76970		

C57BL/6J 1000mg/L

SOURCE	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARES	F VALUE	TAIL PROB.
MEAN	246758.54816	1	246758.54816	516.36	0.0000
TREAT	26.95655	1	26.95655	0.06	0.8137
FMAT	20.51396	1	20.51396	0.04	0.8371
SEX	3.23089	1	3.23089	0.01	0.9350
TF	513.72783	1	513.72783	1.08	0.3074
TS	574.48451	1	574.48451	1.20	0.2808
FS	117.22047	1	117.22047	0.25	0.6237
TFS	414.68314	1	414.68314	0.87	0.3583
ERROR	15770.05048	33	477.88032		
MIN	4795.82305	4	1198.96084	10.03	0.0000
MT	488.82305	4	122.20576	1.02	0.3909
MF	643.87757	4	160.96939	1.35	0.2616
MS	165.86558	4	41.46639	0.35	0.8117
MTF	732.00422	4	183.00105	1.53	0.2067
MTS	244.87821	4	61.21955	0.51	0.6938
MFS	426.19608	4	106.54902	0.89	0.4569
MTFS	1394.94296	4	348.73574	2.92	0.0325
ERROR	15784.39714	132	119.57877		

Median (+ lower and upper intraquartile ranges) values of bouts of *crawling* behaviour by C57BL/6J pups following prenatal exposure to oral aluminium sulphate (750mg/L, Gd10-16).

DURATION

Day	Cc	Ct	Tt	Tc
6	19.8 (4.2, 29.8)	16.4 (0.0, 43.5)	38.9 (24.2, 65.6)	33.0 (7.8, 62.4)
9	74.3 (44.6, 109.7)	45.8 (23.1, 60.4)	65.8 (42.2, 88.5)	60.4 (40.1, 81.1)
12	41.7 (28.5, 53.7)	48.9 (33.7, 63.7)	35.1 (15.4, 55.7)	54.8 (38.6, 77.9)
15	32.6 (10.4, 47.7)	56.6 (17.4, 97.7)	49.7 (28.6, 68.7)	52.5 (14.2, 83.2)
18	118.1 (87.6, 143.5)	129.7 (111.8,148.8)	117.7 (97.8, 150.2)	123.1 (107.7, 144.2)

FREQUENCY

Day	Cc	Ct	Tt	Tc
6	2.0 (0.8, 4.3)	0.0 (0.0, 3.5)	3.0 (1.0, 7.0)	3.0 (1.0, 6.0)
9	9.0 (5.8, 10.5)	6.0 (4.0, 8.0)	7.5 (4.8, 13.0)	11.0 (5.0, 13.0)
12	8.5 (4.5, 9.3)	8.0 (5.5, 13.0)	7.0 (11.5, 17.5)	11.0 (6.0, 13.0)
15	6.5 (3.0, 14.0)	15.0 (19.5, 29.0)	19.0 (19.0, 27.0)	18.0 (7.0, 29.0)
18	28.5 (22.3, 32.0)	26.0 (20.0, 30.0)	23.0 (19.8, 31.0)	25.0 (21.0, 26.5)

HEADUP

Day	Cc	Ct	Tt	Tc
3	2.0 (1.0, 3.5)	4.0 (3.0, 5.0)	4.0 (3.0, 9.0)	4.0 (2.0, 4.0)
6	7.0 (5.8, 9.3)	5.0 (3.5, 5.5)	7.0 (5.8, 9.0)	7.0 (4.0, 9.0)
9	9.5 (7.0, 12.5)	9.0 (6.0, 10.0)	8.5 (7.0, 13.0)	9.0 (7.0, 13.0)
12	11.0 (9.5, 13.5)	12.0 (10.0, 13.0)	11.5 (8.0, 17.3)	14.0 (8.0, 17.0)
15	11.5 (9.8, 19.0)	16.0 (11.0, 26.5)	17.0 (8.0, 23.0)	19.0 (12.0, 27.0)
18	22.0 (19.3, 26.0)	13.5 (12.0, 16.3)	15.0 (12.8, 19.3)	17.0 (11.5, 20.5)

CORRECT RESPONSES

SOURCE	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARES	F VALUE	TAIL PROB.
MEAN	1997.36967	1	1997.36967	199.55	0.0000
TREAT	3.56787	1	3.56787	3.93	0.0730
CHALL	13.11742	1	13.11742	14.45	0.0029
TC	0.50480	1	0.50480	0.56	0.4715
ERROR	9.98889	11	0.90808		
DAY	1.21141	2	0.60571	0.83	0.4332
DT	2.36456	2	1.18228	1.61	0.2264
DC	1.16096	2	0.58048	0.79	0.4465
DTC	3.39520	2	1.69760	2.32	0.1330
ERROR	16.11111	22	0.73232		

APPENDIX H

BEHAVIOURAL EFFECTS OF GESTATIONAL EXPOSURE TO ALUMINIUM.

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Summary. - The involvement of aluminium in the aetiology of a number of human pathological diseases has altered its status from being a nontoxic, nonabsorbable, harmless element. This maybe of particular concern to the developing foetus which is more susceptible to agents and at lower levels than the adult. Little attention has been given to aluminium's potential reproductive toxicity until recently and further research is required for a full evaluation of its toxicity. Our preliminary results demonstrate behavioural and neurochemical alterations in the offspring of mice exposed to aluminium during gestation. Further, the effects of such exposure are also present in the adult animal suggesting persistent changes in behaviour following prenatal exposure.

KEY WORDS: Aluminium Prenatal exposure Mouse

Riassuto ( ). -

Introduction

Human exposure to aluminium (Al) arises from several sources; it is present in food and used in the food processing industry, in cookware, in pharmaceutical preparations and as a flocculant in water treatment. In addition, there are growing amounts of Al entering the environment, particularly in the area of Al smelters, and as a result of leaching from soils by acid rain [1]. From these diverse routes of contact there is an increased likelihood of unknowingly being exposed to Al at levels higher than is recommended.

For a long time it had been presumed that exposure to Al was without toxic effect. However, in contrast to this assumed harmlessness, Al has been implicated as a causative factor in several human pathological diseases in recent years; dialysis encephalopathy and osteomalacia [2], senile dementia of the Alzheimer's type (SDAT) [3], amyotrophic lateral sclerosis and Parkinson's disease of Guam [4]. Although its exact role in these diseases is still a subject of discussion, this implication has led to an abundant literature concerning possible mechanisms by which Al exerts its neurotoxicity in adult animals and to tentative animal models for its effects on human beings.

The lack of concern over the effects of Al ingestion resulted from the assumption that absorption from the gastrointestinal tract was minimal. It is now known that this is not the case and that Al may be absorbed e.g. from Al containing antacids [5]. Moreover, the absorption of Al is increased by the presence of parathyroid hormone [6] and by certain dietary factors, particularly citrate [7].

Of greater relevance to behavioural teratology is the accumulating clinical evidence which points to Al loading in infants with renal dysfunction not receiving dialysis but exposed to Al from the oral ingestion of Al-containing antacids. The levels of Al in plasma, serum and bone were elevated compared to controls [8,9]. Moreover, Sedman *et al.* [10] have shown that the intravenous feeding of premature infants resulted in a 10 fold increase of Al in bone. Further, urinary Al concentration did not reach the control level until several weeks after parenteral feeding was stopped, suggesting an accumulation of Al within body tissues. In addition, cases have been reported of infants with Al intoxication when the only possible source of exposure was from infant formula [11].

In the only documented study of accidental exposure of pregnant women to high levels of oral Al, Golding and coworkers [12] found no effects on the occurrence

of perinatal death, on body weight at birth, before term delivery or congenital malformations. However, there was an increased incidence of talipes among exposed infants (4.4% of cases).

In contrast to the extensive literature on the neurotoxic effects of Al, few studies have considered the consequences of exposure to Al compounds during pregnancy and the early postnatal period. In recent years some attention has been given to such effects on the developing foetus but a full evaluation of risk effects has not been undertaken. This is of particular concern as exposure of the developing nervous system to insults may have very different consequences than those resulting from exposure of the adult nervous system. Furthermore, the developing foetus is more susceptible to and affected at doses far lower than those required to produce changes in the adult central nervous system [13].

The paucity of studies aimed at assessing this aspect of Al's biology may have resulted from the lack of detailed information on the distribution of Al following exposure and from the lack of adverse effects revealed by initial studies [14]. Within the studies which have addressed this question, it is difficult to generalise the effects resulting from exposure to Al. This is because studies have used different Al salts, the solubility of which vary greatly [15], different doses and experimental animals.

In the present paper we briefly report some preliminary findings from our studies on gestational exposure to aluminium sulphate ($Al_2(SO_4)_3$) in mice and compare these with results in other studies. In the frame of our studies aimed at investigating the role of the genotype in individual sensitivity to drugs or toxicants, we have chosen the mouse as our experimental model. This altrical rodent has a similar placentation to that of humans and has been reported to show similarities in behavioural responses following exposure to known human behavioural teratogens [16]. Moreover, the development of inbred strains of mice has led to the introduction of almost isogenic individuals within a strain.

Pregnant CBA mice were exposed to $Al_2(SO_4)_3$ at a dose of 200mg/kg body weight and injected intraperitoneally during days 10 to 13 of gestation. The dose range and gestational period of exposure have been selected on the basis of previous experiments [17,18]. We have used a variety of ethological measures, which have been shown to be sensitive indicators of toxicants, with the aim of assessing subtle behavioural effects on the mother and the behavioural development of pups [19]. The use of a fostering procedure in behavioural teratology studies, to differentiate between direct effects of prenatal exposure and those arising from alterations in maternal behaviour or physiology, has been recommended by a number of authors (e.g. [20]). For this reason, all litters were cross-fostered on the day after birth (postnatal day 1) so that each mother reared two control and two treated pups. This gave 4 treatment groups; control pups fostered to control mothers (Cc), control pups fostered to treated mothers (Ct), treated pups fostered to treated mothers (Tt) and treated pups reared by control mothers (Tc).

Effects of prenatal exposure

Maternal weight

In our experiments gestational weight gain of CBA females was reduced during the treatment period (gestational days 10-13). This decrease in maternal body weight was transient as all mothers increased their weight following the termination of treatment. Further, there was no difference in body weight between the treatment groups during the preweaning period. A decrease in maternal body weight following exposure to oral and injected Al has been observed by several authors [21,22].

Maternal behaviour

Alterations in the behaviour of the mother are known to affect infant development and several drugs have been shown to disrupt elements of maternal behaviour [23]. Thus, any disturbance to maternal care or the delicate mother-pup relationship may explain differential patterns of behaviour in the offspring rather than direct effects of prenatal exposure to a toxicant. The results of a pilot study suggested that differences exist in the pattern of maternal behaviour displayed by control and Al-exposed mothers. Control mothers spent more time involved in the pup-directed behaviours of nursing and licking and less time in nest-building during the first two postnatal weeks than dams treated with Al during gestation.

To further characterise any differences between Al-treated and control mothers we employed a pup retrieval test. Treated mothers had a longer latency to retrieve on postnatal day 3 (Pd3), although this difference did not reach significance.

Only one previous study has included simultaneous recording of maternal behaviour after prenatal exposure to Al, showing no significant differences between treatment groups in nest-building, retrieval of pups to the nest or time spent with the young following exposure to $AlCl_3$ in the dam's diet from day 8 of gestation [24].

Thus it will be necessary to quantify further the maternal behaviour of mothers rearing control and Al-treated pups, an investigation currently being undertaken in our laboratory.

Pup weight

Prenatal exposure of pregnant female CBA mice to $Al_2(SO_4)_3$ had no significant effect upon breeding performance; the length of gestation, litter size and sex ratio were unaffected by prenatal Al. Pup mortality, as a result of infanticide or neglect by the mother, occurred in both treatment groups at birth, thus this cannot be attributed to Al. The mean birth weight was significantly lower (by 6%) among Al-exposed offspring.

Similarly, the offspring of BALB/c mice had a reduced birth weight after i.p. exposure to $AlCl_3$ during gestation days 7 to 16, and an increased incidence of foetal resorptions was also reported [25]. Such effects on Al-treated offspring were also found following oral exposure of pregnant Sprague-Dawley rats to Al by gavage [26]. The lower birth weight of CBA treated pups persisted for those pups reared by treated mothers only, reflecting the significant effect foster mother treatment had on body weight. Thus, when treated pups are given adequate maternal care it is possible to overcome the weight impairment. As maternal food and water intake did not differ during the preweaning period, the diminished body weights cannot be accounted for in terms of nutritional deficiency.

This postnatal maternal influence may have resulted from retention of Al within the mother's body allowing continued exposure via the dam's milk after termination of treatment. Yokel and McNamara [27] injected lactating rabbits with aluminium lactate (AlLact) and found 12% of the total injected Al still present in the area of the injection site seven days after the last injection. This postnatal effect seems likely as in our experiment control pups, which had no prenatal contact with Al, fostered to treated mothers (Ct) had reduced body weights compared to control pups reared by control mothers (Cc). Yokel [21] also found this to be the case; offspring of mothers exposed to 400 umol/Al/kg in utero and control offspring fostered to these does, gained less weight.

Impairment in offspring body weight gain during the preweaning period is the most consistent observation following prenatal exposure to Al, irrespective of the route of administration or the chemical form of Al used [21,22,24,26]. However, the extent of present research with gestational Al does not permit the exclusion of a maternal factor accounting for this weight impairment.

Neurobehavioural development

We have employed a modified version of the Fox battery of tests to measure sensory-motor development in control and Al-exposed offspring [28]. We found that treated offspring differed in their attainment of a mature response for forelimb grasping and pole grasping on Pd15. A maternal effect appeared also in the results for pole grasping as few Ct or Tt pups had reached a mature response by Pd15. Similarly, a significant difference between treated and controls was found for slow righting on Pd6, cliff aversion on Pd12 and screen climbing on Pd18. The impairments in attainment of these reflexes were transitory as all pups eventually achieved the adult response.

It could be argued that the delays in neurobehavioural development are secondary to the reduction in physical maturation. However, we did not find a correlation between the performance in the particular Fox measure and body weight on the same day. Bernuzzi *et al.* [24] found that by Pd9, when delays in the response to negative geotaxis were present, no further treatment group differences in body weight existed. In accordance with our observations, Golub *et al.* [22], using a combined score to estimate performance in a battery of neurobehavioural tests, observed a delay in prenatally Al-treated offspring on Pd14 and 16.

Thus, Al treatment *in utero* affects maturation of certain sensory-motor skills but not all.

Pup behaviours

In our study with CBA pups, the overall expression of the behavioural repertoire was unaffected by exposure to Al. However, the appearance of certain behaviours were delayed in Al-exposed offspring. Pup locomotor activity during postpartum days 9-18 showed a trend towards diminished activity levels in pups reared by treated mothers. Control pups spent more of their time involved in crawling compared to treated pups, as was the case for pups fostered to control mothers. In line with this, pups fostered to treated mothers exhibited a greater frequency of bouts of being still. On Pd18 treated pups exhibited a greater number of bouts of rearing which were of longer duration than that of controls.

Ultrasonic vocalisations

Rodent neonates emit ultrasonic vocalisations with a reliable and stable pattern from a few days after birth. The developmental and physical characteristics of such calls depend not only on the species under investigation but also the strain. As pointed out by Zbinden [29], this response may function as a sensitive indicator of subtle behavioural effects caused by prenatal or postnatal drug treatments. Several authors have shown consistent alterations in the production of these calls following drug exposure which have led them to recommend their incorporation in any battery of tests aimed at assessing behavioural teratogenicity [30].

In a very recent study we found that prenatal treatment with Al produced a striking reduction in the total number of ultrasonic vocalisations emitted by exposed CBA pups compared to controls on removal from the nest [31]. This difference was especially pronounced on days 3 and 4 after birth. In addition, treated pup calling did not reach a maximum until a day later than control offspring. In combination with the results of the sensory-motor tests, in which pups exposed to Al prenatally were slower to mature in certain tests than controls, this suggests that the mechanisms responsible for sound production matured later in Al-treated offspring. An alternative explanation is that *in utero* exposure to Al alters the ability of the neonate to detect or respond to a reduced temperature.

It is not known if the ultrasonic calls of Al-exposed pups differed in other acoustic properties e.g. sound pressure, duration etc. as such measures were not

undertaken. However analysis of such characteristics, to further reveal any treatment effects, are currently under investigation.

Activity at weaning

The effect of *in utero* exposure to Al on CBA activity scores in an openfield at weaning (Pd21) was sex dependent. Treated females had lower activity scores during the 5 minute test than control females. Conversely, Al-exposed male pups crossed more squares than controls. Although treated pups took less time to approach a novel object placed in the centre of the openfield at the end of the activity test, this difference was only marginally significant.

In a test of locomotor coordination, in which the subject was placed in water and could reach a platform by climbing a metal rod, Wistar rat pups from mothers treated orally with AlLact during gestation required more time to complete the task [32].

Long-term effects of early exposure

Since subtle behavioural changes are not always immediately apparent, the attribution of such an effect to a substance taken by the mother during pregnancy is difficult. Thus, in experimental models of behavioural teratology it is vital to test subjects at different ages throughout life to assess whether effects from exposure are transient and may be recovered from, persist into adulthood or are present as delayed effects which only arise late in development. To date, few studies have considered long-term effects of early Al exposure on the adult animal. In our experiments, 12-week old male subjects were tested in an 8-arm radial maze to assess learning ability. Results suggested an effect of Al on performance in the maze. More control animals reached the criterion of eight correct arm choices in the first 8 entries on 2 consecutive days (77%) than subjects previously exposed to Al *in utero* (55%). Also, the number of days required to reach this criterion was greater for treated mice. Tsujii and Hoshishima [33] observed deficits in learning ability of 5- to 7-week old mice exposed prenatally to repeated injections of Al and raised in standard laboratory cages. Young rabbits exposed during gestation to Al and tested for a classically conditioned response task at 11 weeks of age, showed a dependent effect of dose; animals in the low dose group performed better whilst the high dose group showed impaired acquisition and retention [21]. Muller *et al.* [32] found that the performance of rats treated prenatally with AlLact in an operant conditioning test, carried out 65 days after birth, was behind that of controls. In an activity test performed at 22 weeks of age, CBA female mice reared by treated mothers (Ct and Tt subjects) tended towards lower activity scores.

In our experiments, the impairment in growth of offspring reared by treated mothers persisted into adulthood. As with performance in a classical conditioning learning task, Yokel [21] found a biphasic effect of gestational exposure to subcutaneously injected AlLact on the offspring's weight gain after weaning. Although the results were not significant, exposure to low levels of Al increased weight gain whereas weight loss was recorded at high exposure levels.

Neurochemical changes

The most consistent neurochemical finding in postmortem brains of patients with, for example, SDAT, has been alterations to the cholinergic system, the extent of which varies with brain region [34]. Because of the suggested association between the incidence of these diseases and exposure to Al, we have measured the levels of choline acetyltransferase (ChAT), the enzyme responsible for the synthesis of acetylcholine, in a number of brain regions and at different ages.

Although some inconsistencies existed in the levels of ChAT activity at each age and between each brain region, the direction of change in the cerebral cortex, cerebellum and hippocampus (the areas affected in SDAT) between control and treated subjects was the same at each time point; treated brains exhibited a reduced level of enzyme activity compared to controls. For example, the amount of ChAT activity measured at 34 weeks of age in the cerebral cortex of treated animals was 44% less than that in controls.

Discussion

It is clear that there is a lack of information on a number of behavioural measures following gestational exposure to Al, which limits a more comprehensive evaluation of aluminium's behavioural teratogenicity. The results presented in this brief report suggest that prenatal exposure to Al causes alterations to body growth, ultrasound production and sensory-motor development. Moreover, exposure to Al during gestation may result in behavioural deficits during early postnatal development which may persist into adulthood.

The foetus may be directly exposed to Al *in utero* via placental transfer. The extent of any persistence of Al in the body is not fully known. However, the data presented herein suggests that Al is available to distribute into the dam's milk resulting in continued exposure of the neonate over time.

Further work is required to investigate the consequences of Al exposure on the mother-infant interaction. For example, as ultrasonic calls function to elicit maternal care [35], exposure to Al during gestation may have affected the ability of treated mothers to respond adequately to the ultrasonic calls of their fostered pups.

Although the exact neural mechanisms responsible for eliciting ultrasonic vocalizations are unknown, the involvement of a number of different neurotransmitter systems has been proposed. These include the dopaminergic [36], GABAergic [37], cholinergic [38] and the opioid system [39]. We have shown that prenatal exposure to Al resulted in decreased levels of ChAT during adulthood which leads to the tentative suggestion that the cholinergic system may play a role in the production of these calls.

From a methodological point of view the results we report herein certainly suggest that ultrasound recording should be included in any battery of tests aimed at assessing possible behavioural teratogenicity.

In particular, the presence of a maternal influence in our results emphasis the importance of including a fostering design in teratology studies to ensure adequate interpretation of results. To date only one study has done so [21].

Al is present in the environment in a number of different chemical forms. The extent of the solubility of Al in the body is dependent on the Al salt to which exposure results, thus the need to evaluate inter- and intraspecies differences in the bioavailability of Al and its different salts is great. As susceptibility to a number of drugs depends on the genotype, further studies of gestational exposure to Al with a second inbred strain of mouse are being carried out.

Aluminium may be poorly absorbed from the gastrointestinal tract under normal conditions. However in the developing organism such protective barriers are immature which may render it more susceptible to any toxic effects.

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