Animal models: their role in understanding brain dysfunction

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Over four decades ago in 1968 a group of French physicians described a cluster of features that they had found occurred only in children born to alcoholic mothers (Lemoine et al. 1968). Several years later a very similar set of symptoms was recognised and the term 'Fetal alcohol syndrome' (FAS) became a clinical entity to describe the offspring of women (Jones & Smith 1973; Jones et al. 1973). FAS is a well recognised syndrome characterised by three key features: pre- and post-natal growth retardation; a characteristic pattern of facial dysmorphology; and some manifestation of central nervous system dysfunction. All three features must be present for a diagnosis of FAS. Despite an increased awareness of the dangers of drinking during pregnancy, FAS is still considered to be the leading known preventable cause of mental retardation (Abel 1995). There is an increasing number of children and adults with this diagnostic label and the more recently described fetal alcohol spectrum disorder which encompasses all individuals with disabilities due to exposure to alcohol during fetal development (May et al. 2009). Many of these cases are part of prospective research projects investigating both brain structure and function (Driscoll et al. 1990; Mattson & Riley 1998).

When FAS was first described, ethanol was not generally considered to be a teratogenic substance and the pregnant alcohol-consuming women often exposed the developing fetus and child to a very complex environmental experience including exposure to other drugs of abuse, compromised nutrition both pre- and post-natally and suboptimal postnatal care. Thus it was considered that the observed syndrome could have many contributing causes. It was crucial to determine whether alcohol was a teratogen and this initiated the use of animal studies to investigate the effect of alcohol on development.

When considering studies that use an animal model it is important to consider what is meant by the term "animal model". Generally a model is a means of best representing the essential features of a particular object, event or scenario. An animal model therefore is the use of an animal to mimic some scenario that occurs in a human. It is also important that data obtained using an animal model can be extrapolated to the human situation. The first studies involving the use of animal models were designed to investigate whether alcohol was teratogenic and able to produce the physical features that characterised the initial groups of children diagnosed with FAS (Webster et al. 1980; Sulik et al. 1981; Sulik & Johnston 1983; Webster et al. 1988). These studies provided the essential evidence that ethanol, even a single binge-like exposure, could result in alterations in development that mimicked those seen in children diagnosed with FAS and was therefore a teratogenic substance.

A plethora of animal models have been developed over the last three decades to investigate various aspects of the effects of alcohol on the development of the fetal brain, skeletal structure, a range of organ systems and behaviour. The animals used include a number of rodent species, the sheep, miniature swine, non-human primates and also some non-mammalian species such as the chicken, frog and increasingly zebra fish. The essential feature of all these studies is that the question investigated is particularly relevant to

study in the specific species used as the animal model and is a question that cannot be answered in human studies. The laboratory rat is commonly used as the animal of choice in many studies of FAS disorder (West 1986; Goodlett et al. 1991; Kelly et al. 2009). Reasons for this are that there is a large amount of data available on the basic anatomy, physiology and pharmacology of the rat brain. This combined with its short gestation and extreme intelligence make the rat particularly useful in studies where the aim is to correlate changes in brain structure with function. There are also a large number of different rat strains available for use in investigating the relationship between genotype and ethanol fetal sensitivity (see Cudd (2008) for a recent review of animal models use for studying fetal alcohol exposure). The findings in animal model studies correspond well to findings in human studies thus validating the use of rats in FAS disorder research (Driscoll et al. 1990).

One of the key features to be determined in this work has been the sensitivity of the developing nervous system to alcohol. There are now many clinically based studies that have investigated brain structure, using real time in vivo imaging methods such as magnetic resonance imaging and brain function via performance on behavioural tasks (Mattson et al. 1998; Roebuck et al. 1998; Mattson et al. 2001). Despite this, our knowledge of both the amount of alcohol and the frequency and timing of alcohol exposure required to induce damage in the developing brain is rudimentary. It is very difficult to obtain reliable data about the amount of and timing of alcohol exposure from the birth mother of a child diagnosed as alcohol-affected, because exposure likely occurred a year or more before the realisation that the child has been affected by alcohol. Thus the use of animal models is crucial if we are to understand the risk factors involved in alcohol-induced damage and the long-term outcomes. This is particularly important when considering the deleterious effects of exposure to alcohol later in fetal development as the important indicator, the characteristic pattern of dysmorphology seen in the face, is absent (Wattendorf & Muenke 2005) .

Although the majority of women abstain from alcohol consumption during pregnancy there are a number of women who continue to drink and many of this group consume alcohol irregularly but in a binge-like manner (Watson & McDonald 1999). This binge-like consumption pattern, a large number of standard drinks over a short time interval, is of extreme importance as it results in a high blood ethanol concentration. Acute and long-term neuronal deficits in a number of brain regions increase as the peak blood ethanol concentration increases (Bonthius & West 1990; Thomas et al. 1996).

Dietary monitoring in a cohort of New Zealand women during the second trimester of pregnancy found that binge drinking episodes occurred in a range from infrequently, once per month, to up to four times per week (Watson & McDonald 1999). The very first studies using animal models showed conclusively that a single binge exposure to alcohol could alter development and offspring showed a reduced forehead width suggestive of reduced brain damage (Sulik et al. 1981). Few studies have investigated the consequences of a single binge ethanol exposure on fetal outcome later in development. Those studies that have, have used very high alcohol doses within a binge delivery paradigm which has resulted in very high peak blood alcohol concentrations (BACs) which would mimic severe human binge drinking rather that a more social drinking paradigm as used in the present study (Thomas et al. 2001; Thomas et al. 2004). It is not known whether a single binge consumption of alcohol at more moderate doses, with more moderate peak BACs, as would be seen in regular social drinking women, is deleterious to brain development resulting in structural brain changes and subsequent functional deficits in the offspring.

I will use one example from studies carried out in our laboratory to illustrate how using a rat model can inform us on the potential risks of exposing the fetal brain to a single binge exposure to alcohol later in development. This study investigated the dose response effect of a single alcohol binge on the structure and function of the hippocampus, a brain region critically involved in learning and memory. The developing rat brain was exposed to alcohol on a single day, postnatal day 6, during early neonatal life, which is equivalent to exposing the fetal human brain to a single binge exposure during the middle of the third trimester of pregnancy. Postnatal day 6 was selected as the developing hippocampus is particularly vulnerable to the toxic effects of ethanol at this stage of development (Thomas et al. 2001).

Methods

Subjects

Long Evans rats obtained from the University of Otago Breeding Unit (Otago, New Zealand) were maintained under standard housing conditions with a 12-hour light/dark schedule. All manipulations were approved by the University of Otago Animal Ethics Committee. Pups were obtained from timed pregnant dams to ensure age of animal used was equivalent in all groups.

On postnatal day 6 (PD 6), pups within each litter were assigned to one of 5 treatment groups; ethanol intubation at 4.5 g/kg ethanol/day (E4), 5.25 g/kg ethanol/day (E5), or 6.0 g/kg ethanol/day (E6); sham intubation (IC), or unintubated control (UC). The alcohol dose was delivered in an artificial milk solution (Intralipid, intravenous infusion, Baxter Healthcare Pty Ltd. Fresenius Kabi AB) via intragastric intubation with IC animals received a sham intubation, only and UC animals were suckle fed by the dam (Goodlett et al. 1998). A 20 µl blood sample was taken from all E and IC animals, 90 minutes after the last ethanol delivery. This sample was analysed using gas chromatography to determine the peak blood ethanol concentration in the pup (Bonthius & West 1990). This ethanol administration regimen mimics the ethanol blood profile that would be expected from a binge-drinking pregnant woman (Watson & McDonald 1999).

Behavioral Morris Water Maze (MWM) Testing

The animals were tested in the *reference memory version* of the MWM, beginning on PD 111/112. Each animal was subjected to 4 trials per day, with an inter-trial interval of 8 minutes, on 4 consecutive days with the starting position decided in a pseudorandom order. In each trial the animal was allowed to swim for a maximum of 60 seconds and remained on the platform for 10 seconds. On day 5 each animal underwent a single *probe* trial, 60 seconds long, with no platform present. On day 6 a *visual cued platform test* was carried out to verify that there were no group differences in the rat's ability to identify the platform, swim to it and climb on. There was no significant difference between any of the 4 groups tested with all animals able to locate the visible platform, swim to it, and climb on. (Data are not shown.)

Stereological determination of CA1 pyramidal cell number

On PD 290 animals were deeply anesthetised (sodium pentobarbital, 100 mg/kg i.p) and transcardially perfused with heparinised phosphate buffered saline (PBS, pH 7.2), followed by 4% paraformaldehyde in PBS. Brains were removed, frozen and a systematic random series of coronal sections (60 µm thick) was collected and stained through the hippocampus. Using an Olympus AX70 light microscope the total number of pyramidal neurons within the CA1 region of the hippocampus was counted using the fractionator method (West et al. 1991).

Statistical analysis

Cell counts were analysed using one-way ANOVA with treatment group as the factor (Graph Pad Software Inc). For the MWM, mean distance travelled per day was analysed using two-way ANOVA with the between subject factor as treatment, and the within subject factor as day. A significant result in the ANOVA was analysed with Bonferroni post-tests. In the probe trial, the percentage of path length in each of the four quadrants was analysed using one-way ANOVA with treatment group as the factor.

Results

There was a dose-dependent depletion in the total number of pyramidal cells in the CA1 region of the hippocampus. One-way ANOVA indicated a significant effect of treatment on the mean number of pyramidal cells [$F_{(3,28)} = 22.21$, $P < 0.0001$]. All alcohol groups had a significant reduction in CA1 pyramidal cells compared with the control group with the E6.0 group also having significantly fewer cells than the E4.5 group (Figure 1).

A single binge alcohol treatment on PN6 had a significant dose-dependent effect on the mean path length in the reference memory task ($F = 8.57$; $P < 0.001$). Post-hoc comparison revealed significant differences between the alcohol-exposed animals and the sham intubated control animals and within the alcohol-treated animals in a dose-dependent manner on both day 1 and day 4 of training. On day 1, the mean path-length for both E6 and E5 animals was significantly longer than for E4 and IC ($P < 0.001$) with no significant difference between E4 and IC. On day 4 the mean path-length travelled to the platform

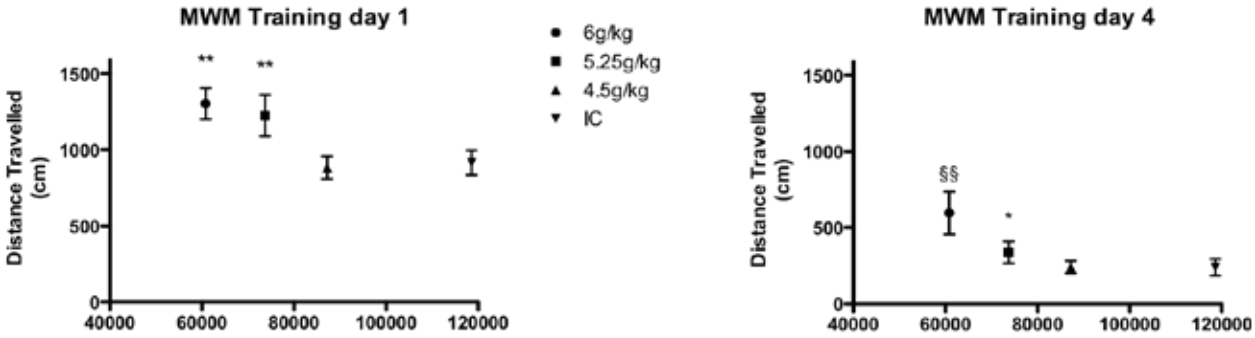

Fig 1 Acquisition of spatial navigation in the Morris Water Maze was impaired on both training days 1 and 4 by a single binge ethanol exposure on PD 6, in a dose-dependent manner. ** $P < 0.001$ vs. 4.5 g/kg (E4) and IC; §§ $P < 0.001$ vs. 5.25 g/kg (E5), 4.5 g/kg (E4) and IC; * $P < 0.05$.

by E6 was significantly longer than for E5, E4 and IC ($P < 0.001$). The mean path-length travelled by E5 was significantly longer than that travelled by animals in groups E4 and IC 4 ($P < 0.05$) (Figure 1). There was no significant effect of treatment on quadrant preference in the probe trial ($F = 0.43$; $P = 0.733$). These data indicate that although the animals in the high dose alcohol group had learned the general position of the hidden platform their strategy to locate it was much less efficient than for all other groups.

Discussion

This study, using the Long Evans rat model of FAS disorder, has shown that a single binge ethanol exposure on PN6 results in a permanent deficit in the total number of pyramidal cells in the CA1 region of the hippocampus. This was accompanied by spatial learning deficits on a hippocampal-based task.

It is particularly important to note that at all alcohol doses used in this study, exposure of the developing brain only once during development resulted in significantly less CA1 pyramidal neurons than in the sham intubated control animals. This indicates that exposure, even a moderate peak blood ethanol concentration, is sufficient to induce a permanent deficit in this population of neurons if exposure occurs during a period of development where the cells are particularly vulnerable. Performance on the reference memory version of the Morris Water Maze does not mirror this cell loss exactly as the E4 alcohol-exposed animals, despite a significant deficit of CA1 cells, do not show a reduced performance on this spatial learning task. However, animals exposed to the two higher ethanol doses showing deficits on training day 1 and despite some improvement in performance, still showed a significant performance deficit at the end of training on day 4. It appears that there is a minimum number of neurons required to carry out the task at normal performance levels and when cell number drops below this, although learning does occur, as evidenced by both day 4 reference memory data and the probe trial data, performance is impaired.

Performance on the probe trial is used as a measure to indicate whether spatial learning has occurred and on this measure there was no impact of alcohol exposure. Although alcohol-exposed animals could perform the task, the data from the training trials indicate that the efficiency of alcohol-exposed animals to carry off the task is significantly less than for animals with no developmental alcohol exposure. Future studies will investigate whether additional days of training improve task performance in the alcohol-exposed animals.

These data have implications for understanding the effects of intermittent drinking at moderate levels during pregnancy. There is no reason to believe that CA1 pyramidal cells in the developing human brain will behave differently to rat CA1 pyramidal cells when exposed to ethanol at the equivalent stage of development, an idea supported by studies using a sheep model of FAS disorder (Ramadoss et al. 2007). Thus a single binge drinking session by a pregnant woman could result in a significant loss of neurons in the hippocampus of the offspring that may impact on their ability to perform learning and memory tasks. This is an important finding because when exposure to ethanol occurs during mid or late gestation, the facial dysmorphology characteristic of FAS does not

occur and exposure of the fetus to alcohol, especially if it has been infrequent may not be known. Thus any learning deficits may not be considered to have a neurological substrate. It is also important to note that when damage occurs to the hippocampus during development it is able to compensate and learning and memory deficits are less than would be anticipated. The mechanisms underlying this compensation will be a focus of future studies as they may provide insights into how brain recovery can be enhanced. It is also important to note that the Morris Water Maze, although very specific for testing hippocampal based deficits, is a relatively simple task, and ongoing studies, using more demanding tasks, will be used to investigate the effects of a single binge ethanol exposure on brain development.

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