Chapter-4

Neurotoxicological effect of lead exposure and iron-deficiency: Role of intra-hippocampal cholinergic transplants

Introduction
Methodology
Results
Discussion
**Introduction**

Lead produces a variety of neurochemical deficits which could translate into permanent morphological changes. These changes are reflected as cognitive and neurobehavioural deficits which have been reported in children as well as in experimental animals exposed to lead (Needleman, 1993; Davis *et al.*, 1990). In vivo experiments show a subtle and diffuse pattern of morphological changes in the brain of lead exposed animals, although no specific lesion due to lead exposure has so far been identified (Hasan *et al.*, 1989).

Several reports indicate hippocampus as the primary site of lead toxicity in the brain (Selvin-Testa and Palacios-Pru, 1988; Alfano and Petit, 1982; Bielarczyk *et al.*, 1996) and where the metal has been found to selectively accumulate (Fjerdingstad *et al.*, 1974; Schenhammer and Cherian *et al.*, 1982). Alterations in both choline and acetylcholine levels, evoked and potassium stimulated cholinergic release, acetylcholine turnover rates, acetylcholinesterase and choline acetyltransferase activity in lead treated animals and muscarinic receptor binding suggest impairment of the
cholinergic function (Bielarczyk et al., 1996; Alfano and Petit, 1985; Cory-Slechta and Pokora, 1995; Bondy and Agarwal, 1980; David and Tomas, 1995; Dorothy and Dorothy, 1984).

The hippocampus occupies a key position within the limbic system, with diverse and widespread inputs reaching into it via the entorhinal cortex. There are also reciprocal connections between the hippocampus and the adjoining brain regions mainly using the entorhinal cortex as the interface (Isaccson, 1987, 1982). Thus, the hippocampus is an area which is integrating and combining stimuli of different sensory fields as well as comparing the input with stored data from the long term memory (Sprick, 1995).

Since the late 1950's when Scoville and Milner (1957) published the Severe anterograde memory deficit, of their famous patient H.M., which developed after bilateral temporal lobe surgery, it has been accepted to associate the hippocampus with the acquisition of memory. With regard to the consolidation hypothesis which was elaborated by Hebb (1949), the hippocampus seemed to be a structure of great importance for the process of memory storage from reverberating circuits or cell assemblies into the chemically coded long term memory. In recent times another main function of the hippocampal system has been described as the mediation of a capacity for declarative memory, which is that kind of memory supporting conscious recollection and the explicit and flexible expression of memories in humans (Cohen and Eichenbaum, 1994; Eichenbaum, 1992). In rats hippocampal lesions lead to impaired ability to respond in a multi arm radial maze (Olton et al., 1979). Such rats also fail to perform correctly in a Morris water maze (O'Keefe and Nadel, 1978; Jett et al., 1997a,b).

The potency of fetal neural grafts to reduce behavioural impairments after bilateral lesion of the hippocampal formation have been demonstrated by several researchers (Clark et al., 1986; Dunnett et al., 1982; Ridley et al., 1991; Li et al., 1992; Tarricone, 1996). Such grafts have helped to provide cholinergic reinnervation and
thereby recover impairments in cognitive functions in rodent models (Dunnett, 1991; Ridley et al, 1991). Experimental work in animals and to a more limited extent in humans has demonstrated that the cholinergic system is involved in mechanisms which control learning and memory. Since there is cholinergic loss in a variety of dementing illnesses any treatment designed to alleviate the mental symptoms of these diseases, must address the issue of cholinergic dysfunction even if other treatments are also required to overcome other neurotransmitter imbalances. Experimental studies in rodents have demonstrated that cholinergic rich fetal neural transplants can under certain conditions alleviate the behavioural effect of cholinergic lesions or of cholinergic decline (Ridley and Baker, 1993; Hofferer et al, 1996).

Utilization of grafts rich in cholinergic neurons placed into the denervated hippocampus is based on the hypothesis that the cognitive deficits associated with the septo-hippocampal lesions are due to an alteration of the cholinergic component of the septohippocampal pathway (Sinden et al, 1995).

Based on the observation that lead causes cognitive deficits as a consequence of cholinergic hypofunction, and the recent observations of Bielarczyk et al (1996) that the effect of perinatal lead exposure, resemble in several respects, those seen following surgical disruption of the septohippocampal pathway, the present investigation was, therefore, aimed to examine, whether fetal cholinergic rich grafts can restore lead induced functional deficits (to what extent and how early) and the underlying mechanism of neurochemical and behavioural recovery.

Experimental Procedures

Based on the behavioural and neurochemical observations, lead exposed rats and those exposed to lead under iron-deficient conditions were taken for transplantation studies. Each group was divided into two subgroups. One subgroup received intra hippocampal fetal neural suspension rich in cholinergic neurons while the other sub-group was sham operated. Another group which was not exposed to lead and did not receive any transplant served as the control.
Transplantations were done on PND 42 giving a 3 week period between the last lead exposure and neural transplantation. Transplants consisted of cell suspensions (Bjorklund et al, 1983) derived from cholinergic rich basal forebrain primordia of fetuses at 15-17 days gestation. Tissue fragments were pooled and made into a suspension by repeated aspiration through a fine pasteur pipette in lactated Ringer's solution. The final suspension was diluted to obtain approximately 30,000 cells/μl. Two μl of this suspension was injected bilaterally into the hippocampus using stereotoxic coordinates, AP = 2.5 mm, L = ± 3.0 mm and DV = 3.5 mm (Paxinos and Watson, 1982) from Lambda and Dura. Injections were made manually over a period of five minutes with a 10 μl Hamilton syringe which was left in place for an additional 5 minute. Sham operations with the vehicle Lactated Ringer's solution were carried in an identical manner.

Behavioural and neurochemical observations were made 3 and 6 months post transplantation, according to the procedures as described in Chapter 2.

The group differences at 3 and 6 months were analysed using 2 way analysis of variance followed by one way analysis of variance among different treatment groups. Prior to this homogeniety of variance between the treatment groups was ascertained. The significance of mean between the two treatments was analysed by calculating the least significant differences. p values less than 0.05 were considered statistically significant.

Percent restoration was calculated by using the formula:

\[
\frac{C - B}{A - B} \times 100
\]

where A = Control; B = Lead exposed; C = Transplanted.

The formula takes into account the values of both the control and lead exposed animals and therefore gives a better comparison.
Results

General observations

Following cessation of lead exposure the food and water intake of rats improved. No mortality was observed in lead exposed rats, however, 15% mortality was observed in the transplanted as well as sham operated animals. The mortality was only during the first 24 hrs following surgical procedures. Operated animals took 5-6 hrs to recover from anaesthesia and removal of stitches was usually not needed since cage mates would remove these during grooming.

Behavioural observations

Motor deficits as a result of lead exposure were found to persist even upto 7 months post lead exposure. The percent decrease in motor activity was more in iron-deficient lead exposed rats. Transplantation restored motor activity by 45% in the lead exposed rats at 3 months. However, in the iron-deficient lead exposed rats the percent restoration was only 30% at 3 months (Table 7). At the end of 6 months post transplantation, the percent restoration increased and was 60% in lead exposed and transplanted rats and 52% in iron-deficient lead exposed and transplanted rats (Table 8). Other aspects of locomotor function such as ambulatory time, resting time and stereotypic time also showed significant restoration.

Table 7
Effect of intra-hippocampal cholinergic transplants in lead exposed and iron-deficient rats 3 months post transplantation on various locomotor functions.

<table>
<thead>
<tr>
<th></th>
<th>Iron-sufficient (Control)</th>
<th>Iron-sufficient + Pb (Sham)</th>
<th>Iron-sufficient + Pb (Transplanted)</th>
<th>Iron-deficient + Pb (Sham)</th>
<th>Iron-deficient + Pb (Transplanted)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distance travelled (cm)</td>
<td>1535±121</td>
<td>1104±40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1300±50&lt;sup&gt;b&lt;/sup&gt;</td>
<td>748±42&lt;sup&gt;*a,b&lt;/sup&gt;</td>
<td>978±72&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Resting time (sec)</td>
<td>94±8</td>
<td>131±11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>92±4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>151±12&lt;sup&gt;*a&lt;/sup&gt;</td>
<td>142±13</td>
</tr>
<tr>
<td>Ambulatory time (sec)</td>
<td>68±6</td>
<td>50±3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>62±2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>35±2&lt;sup&gt;*a,b&lt;/sup&gt;</td>
<td>47±4&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Stereotypic time (sec)</td>
<td>138±5</td>
<td>119±10</td>
<td>147±3</td>
<td>114±9&lt;sup&gt;*b&lt;/sup&gt;</td>
<td>112±8&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are mean ± S.E. of 5 animals.
<sup>*p <0.05, **p <0.01, a = vs Iron-sufficient (control), b = vs Lead exposed (Sham), c = vs Iron-deficient + Pb (Sham).</sup>
Table 8
Effect of intrahippocampal cholinergic transplants in lead exposed and iron-deficient rats 6 months post transplantation on various locomotor functions.

<table>
<thead>
<tr>
<th></th>
<th>Iron-sufficient (Control)</th>
<th>Iron-sufficient + Pb (Sham)</th>
<th>Iron-sufficient + Pb (Transplanted)</th>
<th>Iron-deficient + Pb (Sham)</th>
<th>Iron-deficient + Pb (Transplanted)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distance travelled</td>
<td>1540 ± 80</td>
<td>1144 ± 62 *a</td>
<td>1282 ± 40 *b</td>
<td>810 ± 24 **a</td>
<td>1190 ± 31 *c</td>
</tr>
<tr>
<td>(cm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resting time (sec)</td>
<td>84 ± 6</td>
<td>147 ± 6 *a</td>
<td>112 ± 5 *b</td>
<td>168 ± 7 **a</td>
<td>122 ± 6 *c</td>
</tr>
<tr>
<td>Ambulatory time</td>
<td>82 ± 4</td>
<td>53 ± 4 *a</td>
<td>68 ± 4 *b</td>
<td>39 ± 4 **a</td>
<td>74 ± 5 **c</td>
</tr>
<tr>
<td>time (sec)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stereotypic time</td>
<td>134 ± 6</td>
<td>100 ± 5 *a</td>
<td>120 ± 5 *b</td>
<td>93 ± 4 **a</td>
<td>104 ± 4 *c</td>
</tr>
<tr>
<td>(sec)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± S.E. of 5 animals.

*p < 0.05, **p < 0.01, a = vs Iron-sufficient (control), b = vs Lead exposed (Sham), c = vs Iron-deficient + Pb (Sham).

Relearning index (RI) as a measure of learning and memory was lower in lead exposed rats even 7 months post lead exposure. Transplantation, however, did not restore the deficit in RI at 3 months post transplantation (Fig. 16). Lead treated and transplanted animals, however, improved in their ability to discriminate visual stimuli under shock stress 6 months post transplantation. Number of incorrect runs were significantly decreased and lead transplanted rats showed a restoration of 57% over controls in relearning index at 6 months, which was 47% in iron-deficient lead exposed and transplanted rats (Fig. 17).

Neurochemical observations

Neurochemical observations after transplantation were encouraging both at 3 and 6 months. AChE activity was restored by 70% in lead exposed and transplanted rats at both time intervals. It was restored by 54% at 3 months and by 66% at 6 months in iron-deficient lead exposed and transplanted rats (Fig. 18).

Cholinergic muscarinic receptor binding was restored by 34% and 47% at 3 and 6 month post transplantation respectively in lead exposed and transplanted rats. In the iron-deficient lead exposed and transplanted rats cholinergic receptor binding was restored by 22% at 3 months and by 38% at 6 months. As revealed by the Scatchard
Fig. 16: Effect of intra-hippocampal cholinergic transplants in lead exposed and iron-deficient rats on shock induced visual discrimination response, 3 months post transplantation. Values are mean ± S.E. of 8 animals. **p < 0.01, ***p < 0.001, a = vs iron-sufficient (control), b = vs iron-sufficient + Pb (Sham), c = vs iron-deficient + Pb (Sham).
Role of intra-hippocampal cholinergic transplants

Percent relearning index

Fig. 17: Effect of intra-hippocampal cholinergic transplants in lead exposed and iron-deficient rats on shock induced visual discrimination response, 6 months post transplantation. Values are mean ± S.E. of 8 animals. *p<0.05, **p<0.01, ***p<0.001, a = vs iron-sufficient (control), b = vs iron-sufficient + Pb (Sham), c = vs iron-deficient + Pb (Sham).
Role of intra-hippocampal cholinergic transplants

Fig. 18: Effect of intra-hippocampal cholinergic transplants in lead exposed and iron-deficient rats on the activity of acetylcholinesterase, 3 and 6 months post transplantation. Values are mean (nmoles x 10^6 of acetylthiocholine iodide hydrolysed/min/mg protein) ± S.E. of 5 animals. 
*p<0.05, **p<0.01, a = vs iron-sufficient (control), b = vs iron-sufficient + Pb (Sham), c = vs iron-deficient + Pb (Sham).
analysis the change in receptor binding was due to an increase in the number of binding sites ($B_{max}$) and not due to a change in the affinity ($K_d$) of the receptors (Table 9).

**Table 9**

Effect of intra-hippocampal cholinergic transplants in lead exposed and iron-deficient rats on cholinergic muscarinic receptor binding 3 and 6 months post transplantation.

<table>
<thead>
<tr>
<th>Time Period</th>
<th>Iron-sufficient (Control)</th>
<th>Iron-sufficient + Pb (Sham)</th>
<th>Iron-sufficient + Pb (Transplanted)</th>
<th>Iron-deficient + Pb (Sham)</th>
<th>Iron-deficient + Pb (Transplanted)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 Months</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$^3$H-QNB</td>
<td>868±31</td>
<td>596±22 $^{**a}$</td>
<td>689±18 $^{b}$</td>
<td>504±18 $^{**a}$</td>
<td>587±19 $^{c}$</td>
</tr>
<tr>
<td>$K_d$ (nmoles)</td>
<td>1.14</td>
<td>1.01</td>
<td>1.17</td>
<td>1.21</td>
<td>1.18</td>
</tr>
<tr>
<td>$B_{max}$</td>
<td>1050</td>
<td>740</td>
<td>850</td>
<td>683</td>
<td>740</td>
</tr>
<tr>
<td>6 Months</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$^3$H-QNB</td>
<td>888±34</td>
<td>684±22 $^{**a}$</td>
<td>780±16 $^{**b}$</td>
<td>591±23 $^{**a}$</td>
<td>704±17 $^{**c}$</td>
</tr>
<tr>
<td>$K_d$ (nmoles)</td>
<td>1.18</td>
<td>0.96</td>
<td>1.04</td>
<td>1.10</td>
<td>1.19</td>
</tr>
<tr>
<td>$B_{max}$</td>
<td>1150</td>
<td>820</td>
<td>1070</td>
<td>738</td>
<td>868</td>
</tr>
</tbody>
</table>

Values are mean ± S.E. of 5 animals for $^3$H-QNB binding. Values are mean of 3 separate set of experiments for Scatchard analysis. $^*p<0.05, ^{**}p<0.01$, $a = \text{vs Iron-sufficient (control)}, b = \text{vs Lead exposed (Sham)}, c = \text{vs Iron-deficient + Pb (Sham)}$.

$Na^+,K^+$-ATPase activity was restored by 57% at 3 months and by 70% at 6 months post transplantation in lead exposed rats. In the iron-deficient lead exposed rats $Na^+,K^+$-ATPase activity was restored by 37% at 3 months and by 58% at 6 months post transplantation (Fig. 19). Monoamine oxidase activity was restored by 38% at 3 months and by 60% at 6 months in the lead exposed and transplanted rats. While in the iron-deficient lead exposed rats MAO activity was restored by 36% and 60% at 3 and 6 months post transplantation respectively (Fig. 20).

Polyamine levels did not show any significant improvement in their levels 3 months post transplantation. However, 6 months post transplantation their levels showed a marginal but statistically significant restoration. The percent restoration was greater in lead exposed and transplanted rats than in iron-deficient lead exposed and transplanted rats (Figs. 21 & 22).

Lead levels at 3 months post transplantation were only 2 fold higher in lead
Fig. 19: Effect of intra-hippocampal cholinergic transplants in lead exposed and iron-deficient rats on the activity of \( \text{Na}^+\text{K}^+ \text{-ATPase} \), 3 and 6 months post transplantation. Values are mean (nmoles Pi liberated/hour/mg protein) ± S.E. of 5 animals. *\( p < 0.05 \), **\( p < 0.01 \), ***\( p < 0.001 \), a = vs iron-sufficient (control), b = vs iron-sufficient + Pb (Sham), c = vs iron-deficient + Pb (Sham).
Role of intra-hippocampal cholinergic transplants

Fig. 20: Effect of intra-hippocampal cholinergic transplants in lead exposed and iron-deficient rats on the activity of monoamine oxidase, 3 and 6 months post transplantation. Values are mean (nmoles of benzaldehyde formed/min/mg protein) ± S.E. of 5 animals. *p<0.05, **p<0.01, a = vs iron-sufficient (control), b = vs iron-sufficient + Pb (Sham), c = vs iron-deficient + Pb (Sham).
exposed rats as compared to the controls and 3 fold higher in iron-deficient lead exposed rats. The levels of lead, however, declined slowly and by the end of 6 months their levels were not statistically significant when compared to the control values (Table 10).

<table>
<thead>
<tr>
<th>Time Period</th>
<th>Iron-sufficient (Control)</th>
<th>Iron-sufficient + Pb (Sham)</th>
<th>Iron-sufficient + Pb (Transplanted)</th>
<th>Iron-deficient (Sham)</th>
<th>Iron-deficient + Pb (Transplanted)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 months</td>
<td>47±12(4)</td>
<td>107±14*8(4)</td>
<td>98±16**8(3)</td>
<td>134 ± 12***8(4)</td>
<td>128±13***8(4)</td>
</tr>
<tr>
<td>6 months</td>
<td>54±15(4)</td>
<td>81±15(4)</td>
<td>76±14***8(3)</td>
<td>90 ± 14(4)</td>
<td>87±12(3)</td>
</tr>
</tbody>
</table>

Values are expressed as mean (ng/g tissue) ± S.E.
Values in parenthesis indicate number of animals.
*p<0.05, **p <0.01, ***p<0.001; a = vs Iron-sufficient (control), b = vs Lead exposed (Sham), c = vs Iron-deficient + Pb (Sham).

Morphological observations

Transplants under the light microscope were observed as a cluster of cells within the hippocampus and with a limited tendency to migrate from their original site of transplant. Although 60,000 cells were transplanted at one site, only 5-10% of the cells were found to have survived (Fig. 23). HRP labelling of the transplant helped to identify transplanted cells, the injection tracts and the placement of the transplant (Fig. 24).

Discussion

Several recent clinical studies have found a significant correlation between lead exposure and learning and behavioural deficits in children (Deborah, 1996; Needleman et al, 1990; Bellinger et al, 1987). The lack of any apparent change in cellular morphology suggest that low level lead induced perturbations of development may reflect an altered neuroplastic state rather than gross cellular lesions, which would lead to functional damage.
**Fig. 21:** Effect of intra-hippocampal cholinergic transplants in lead exposed and iron-deficient rats on the levels of putrescine, 3 and 6 months post-transplantation. Values are mean (nmols/g wet weight of tissue) ± S.E. of 5 animals. *p < 0.05, **p < 0.01, ***p < 0.001, a = vs iron-sufficient (control), b = vs iron-sufficient + Pb (Sham), c = vs iron-deficient + Pb (Sham).
Role of intra-hippocampal cholinergic transplants

Fig. 22: Effect of intra-hippocampal cholinergic transplants in lead exposed and iron-deficient rats on the levels of spermidine, 3 and 6 months post transplantation. Values are mean (nmoles/g wet weight of tissue) ± S.E. of 5 animals. *p < 0.05, **p < 0.01, a = vs iron-sufficient (control), b = vs iron-sufficient + Pb (Sham), c = vs iron-deficient + Pb (Sham).
Fig. 23: Photomicrographs of the hippocampal region of lead exposed iron-deficient rat showing transplant placed dorsal to the dentate gyrus. ax250, bx400 (thionin stained).
Fig. 24: Horse radish peroxidase labelled transplants within the hippocampal region of lead exposed and transplanted rats. The procedure makes it easier to identify transplanted cells, the injection tracts and the placement of the transplant. ax150, bx400 (diaminobenzidine stained).
The special vulnerability of the hippocampal region of developing brain towards the neurotoxic action of lead and subsequent long term learning and cognitive impairments (Alfano and Petit, 1982; Selvin-Testa and Palacios-Pru, 1988) coupled with the recent observations of cholinergic denervation like changes in developmental lead exposed animals which closely resembled surgical disruption of septohippocampal pathway (Bielarczyk et al, 1996), prompted us to investigate if these functional deficits could be restored by cholinergic rich fetal transplant in lead exposed young rats.

Results from the study indicate that fetal cholinergic rich transplants are able to restore the behavioural and neurochemical deficits in lead exposed rats to a significant extent. Although neurochemical deficits improved significantly at both time periods, the restoration in cognitive and motor functions were found to be significant only at 6 months. A possible explanation could be that the elevated levels of lead in the hippocampus could have kept the level of polyamines depressed and, therefore, the low availability of polyamines might have interfered with the proper integration of the transplant with the host brain 3 months post transplantation. Gradually the levels of lead started to decline which was apparent at the end of 6 months and simultaneously polyamine profile changed and their levels increased, thereby enabling the transplanted tissue to elaborate itself within the host brain, further confirming that polyamines are essential for the regulation of cell growth and differentiation (Janne et al, 1991). It was also observed that the restoration was better in lead exposed rats than it was in iron-deficient lead exposed rats. This observation further lends support to the fact that increased amount of lead retention under iron-deficient conditions may interfere with transplant related restoration in these animals.

There is now compelling evidence to suggest that polyamines are potentially involved in the regulation of a number of metabolic and electrophysiological processes because of their functional role in neuronal firing, transmitter release, calcium homeostasis, DNA and protein synthesis (Tabor and Tabor, 1984; Scott et al, 1993; Anand et al, 1976; Iqbal and Koenig 1985; Lopatkin et al, 1994; Bell and Slotkin, 1986).
Recently Adhami *et al.* (1996) reported significant lowering of putrescine levels in the hippocampal glial cells in lead exposed neonatal rats, which could explain the aberration in polyamine regulated macromolecular synthesis and subsequent alterations in the cytoarchitectural development of neonatal brain following lead exposure.

To date several mechanisms of transplant induced functional recovery, some of which may be applicable in the present study, have been proposed. For instance the observed functional effects can also be explained in terms of diffuse release of deficient trophic factors or neurotransmitters, tonic reafferentation of previously denervated areas of host brain or acute trophic influences over recovery phenomena in the damaged host. A minimal integration of the grafted neurons into the host neuronal circuitry may, however, be necessary for certain types of graft induced functional recovery to occur. Thus, the potential of fetal neural transplants to induce or improve behavioural recovery in brain-damaged recipients rests on multitude of trophic, neuro-humoral and synaptic mechanisms that may allow the implanted tissue to influence host brain function or repair mechanisms.

In conclusion grafted cholinergic neurons in lead exposed growing rats appears to have helped to ameliorate the central cholinergic malfunction and anomalies in polyamine release via one or more of the above mechanism(s), thereby facilitating the repair of the perturbed neuronal cytoarchitecture of the hippocampus which is essential for normal cognitive and sensory motor function.