Chapter 5
Neurotoxicological effect of lead exposure and iron-deficiency: Role of intra-cerebellar homotopic transplants

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Introduction

The recognition of the vulnerability of children to lead toxicity, resulting in encephalopathy and permanent neurological sequelae, prompted early investigators to focus on development of an animal model of childhood lead encephalopathy. Pentshew and Garro (1966) produced overt neurotoxicity in suckling pups of rats exposed to a diet containing 40% lead carbonate. In the early 1970s, discussions of lead neurotoxicity in children in the clinical literature used the term hyperactivity. This was appropriately translated by experimental investigators as meaning locomotor activity in animals. Early research in rodents (Silbergeld and Goldberg, 1973, 1974; Sauerhoff and Michaelsen, 1973) revealed increased locomotor activity as a result of high dose developmental lead exposure. There followed a number of reports through the 1970s and into the 1980s focusing on high dose developmental exposure in rodents, with locomotion or similar activity measures as the main dependent variable. Results from these studies were often contradictory suggesting that locomotion was not a sensitive or specific indicator of behavioural toxicity produced by lead (Rice, 1996).
Despite these anomalies, the results indicated that disturbances in neuromuscular ontogeny are sequelae of relatively brief low level lead exposure during development. Lead impairs neuromuscular coordination and alterations in neuromuscular function, both subtle and profound are commonly reported in human and experimental animals following lead toxicity (De La Burde and Choate, 1972, 1975, Pueschel et al, 1972; Landrigan et al, 1975; Repko et al, 1975; Feldman et al, 1977; Overmann et al, 1979; Overmann 1977; Grant et al, 1980; Rice, 1996). Among possible sites of action for the observed effects of lead are: the cerebellum and the motor cortex (Campbell et al, 1970; Takeichi and Noda, 1974; Nathanson and Bloom, 1975; Press, 1977; Tonge et al, 1977; Lotron and Anderson, 1986; Hasan et al, 1989). The cerebellum is involved in maintenance of posture and balance, maintenance of muscle tone and the coordination of voluntary movements (Lorton and Anderson 1986; Triarhou, 1996).

Frequently deficits in fine motor and oculomotor coordination are reported in children and experimental animals suffering from lead intoxication. Lorton and Anderson (1986) reported reduction in granule cell population and decrease in the dendritic arborization of the purkinje cells within the cerebellum of lead treated rats. Press (1977) reported lead to inhibit cerebellar cell proliferation and acquisition, however, Hasan et al (1989) observed that chronic low level lead (45 µg/dl) had no significant effect on cell acquisition or morphogenetic movement within the cerebellar cortex during postnatal development.

Neurochemical deficits have been persistently observed in the cerebellar region of lead exposed animals (Silbergeld and Hruska, 1980). Based on the alterations in various neurochemical parameters due to lead exposure under iron-deficient conditions and the reported reduction of granule cell population and decrease in the dendritic arborisation of the purkinje cells, there may occur loss of synaptic contacts between essential neuronal cells (Hasan et al, 1989). The impaired cell to cell interaction may impair the ability of the cerebellum to interpret and integrate the incoming motor and sensory information and to respond appropriately.
Neuronal transplantation has been successfully applied to replace degenerated neurons in the cerebellum of rats (Triarhou, 1996). Evidence for functional recovery brought about by cerebellar transplants has been presented in the Purkinje cell deficient model of hereditary cerebellar ataxia (Triarhou et al., 1995, 1996; Zhang et al., 1996), in rats with acrylamide induced selective degeneration of purkinje neurons (Husain et al., 1992) and in the weaver mutant mice (Takayama et al., 1987, 1988). Keeping in view the functional role of fetal cerebellar transplants on motor function, the present investigation was carried to study the restorative potential of neural transplants in lead exposed rats and to investigate the underlying mechanism(s) of lead induced neuro developmental deficits.

Methodology

Lead exposed rats and those exposed to lead under iron-deficient conditions were taken for transplantation. Each group was divided into two sub groups. One sub group received intra-cerebellar homotopic fetal neural transplants while as the other sub group was sham operated and received the vehicle i.e. lactated Ringers solution in an identical manner. Another group which was not exposed to lead and did not receive any transplant served as the control.

Transplants were done on PND-42 and consisted of cell suspension obtained from the cerebellar primordia of Embryos (12-15 days of gestation). Several such primordia were pooled and made into a suspension by repeated aspiration through a fine pasteur pipette in lactated Ringers Solution (Triarhou, 1992).

Part of the cell suspension was incubated in HRP (Horse Radish Peroxidase) as a labelling agent to help identification of the transplanted cells. Two μl of the cell suspension containing around 60,000 cells was injected bilaterally into the cerebellum using stereotaxic coordinates, AP = 2.5 mm, lateral = 2.0 mm and ventral = 2.0 mm from Lambda and Dura. Injections were made manually with a 10μl Hamilton syringe over a period of 5 minutes, and left in place for additional 5 minutes to aid diffusion of the suspension within the cerebellum.
Behavioural, neurochemical and morphological observations were made 3 and 6 months post transplantation according to the procedures as detailed in Chapter 2.

Results

General observations

While no mortality was observed in the lead exposed groups, transplanted and sham operated animals showed 15% mortality during the first 24 hours following the surgical operations.

Behavioural observations

Motor function in terms of the total distance travelled as assessed in the optovarimax showed 52% restoration in lead exposed and transplanted animals while iron-deficient lead exposed rats showed a restoration of 39% at 3 months post transplantation (Table 11).

<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>1603 ± 110</td>
<td>1100 ± 92**a</td>
<td>1382 ± 108*a,b</td>
<td>922 ± 88***a</td>
<td>1210 ± 100*a,c</td>
</tr>
<tr>
<td>Resting time (sec)</td>
<td>83 ± 7</td>
<td>127 ± 9**a</td>
<td>92 ± 7**b</td>
<td>145 ± 9***a</td>
<td>123 ± 8c</td>
</tr>
<tr>
<td>Ambulatory time (sec)</td>
<td>59 ± 5</td>
<td>42 ± 5*a</td>
<td>51 ± 4*b</td>
<td>29 ± 3**a</td>
<td>46 ± 3c</td>
</tr>
<tr>
<td>Stereotypic time (sec)</td>
<td>158 ± 9</td>
<td>131 ± 8*a</td>
<td>157 ± 9**b</td>
<td>126 ± 7**a</td>
<td>131 ± 7c</td>
</tr>
</tbody>
</table>

Values are mean ± 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)

The percent restoration at the end of 6 months post transplantation was 93% in case of lead exposed and transplanted animals and 74% in iron-deficient lead exposed and transplanted rats (Table 12).
Role of intra-cerebellar homotopic transplants

Effect of intra-cerebellar homotopic 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 (cm)</td>
<td>1575 ± 115</td>
<td>1058 ± 90***a</td>
<td>1529 ± 105***b</td>
<td>1019 ± 85***a</td>
<td>1424 ± 75***c</td>
</tr>
<tr>
<td>Resting time (sec)</td>
<td>101 ± 8</td>
<td>168 ± 10**a</td>
<td>127 ± 9**b</td>
<td>182 ± 6**a</td>
<td>148 ± 8**e</td>
</tr>
<tr>
<td>Ambulatory time (sec)</td>
<td>54 ± 5</td>
<td>39 ± 5*</td>
<td>48 ± 4**b</td>
<td>24 ± 4**a</td>
<td>38 ± 53**e</td>
</tr>
<tr>
<td>Stereotypic time (sec)</td>
<td>145 ± 9</td>
<td>93 ± 7*</td>
<td>125 ± 6**b</td>
<td>94 ± 8*</td>
<td>116 ± 7**e</td>
</tr>
</tbody>
</table>

Values are mean ± 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)

Other aspects of motor functions such as ambulatory time, resting time and stereotypic time were significantly restored by cerebellar transplants at both time periods. However, the percent restoration was lower in iron-deficient rats (Tables 11 & 12).

Cerebellar transplants failed to elicit any restoration in the learning and memory functions at either time intervals. Transplanted rats did not improve in their ability to correctly respond to a visual discrimination response on a Y-maze (Figs. 25 & 26).

Neurochemical observations

AChE activity was restored by 75% by cerebellar transplants in lead exposed animals at both time intervals. In the iron-deficient lead exposed and transplanted animals a restoration of 61% and 65% was observed at 3 and 6 months post transplantation respectively (Fig. 27).

Cholinergic muscarinic receptor binding was restored by 44% in lead exposed and transplanted rats at both time intervals. In the iron-deficient lead exposed group, transplantation restored the deficit by 27% at 3 months and by 42% at 6 months post transplantation. Scatchard analysis revealed that the increase in binding in the transplanted rats was a result of an increase in the number of binding site (Bmax) and...
Fig. 25: Effect of intra-cerebellar homotopic 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).
Role of intra-cerebellar homotopic transplants

Fig. 26: Effect of intra-cerebellar homotopic 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, a = vs iron-sufficient (control).
Role of intra-cerebellar homotopic transplants

Fig. 27: Effect of intra-cerebellar homotopic 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, ***p < 0.001, a = vs iron-sufficient (control), b = vs iron-sufficient + Pb (Sham), c = vs iron-deficient + Pb (Sham).
there was no significant change in the affinity of the receptors for the ligand (Table 13).

Table 13
Effect of intra-cerebellar homotopic transplants in lead exposed and iron-deficient rats on cholinergic muscarinic receptor binding 3 and 6 months post transplantation.

<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>3 Months</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$^3$H-QNB (nmoles)</td>
<td>1345 ± 105</td>
<td>890 ± 70**a</td>
<td>1090 ± 90**b</td>
<td>760 ± 60**a</td>
<td>918 ± 52**a,c</td>
</tr>
<tr>
<td>$K_d$</td>
<td>1.51</td>
<td>1.15</td>
<td>1.25</td>
<td>0.95</td>
<td>1.10</td>
</tr>
<tr>
<td>$B_{max}$</td>
<td>1775</td>
<td>1008</td>
<td>1245</td>
<td>945</td>
<td>1120</td>
</tr>
<tr>
<td>6 Months</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$^3$H-QNB (nmoles)</td>
<td>1284 ± 110</td>
<td>765 ± 75**a</td>
<td>994 ± 75**a</td>
<td>705 ± 70**a</td>
<td>948 ± 47**a,**c</td>
</tr>
<tr>
<td>$K_d$</td>
<td>1.43</td>
<td>1.05</td>
<td>1.22</td>
<td>0.90</td>
<td>1.27</td>
</tr>
<tr>
<td>$B_{max}$</td>
<td>1640</td>
<td>955</td>
<td>1160</td>
<td>910</td>
<td>1210</td>
</tr>
</tbody>
</table>

Values are mean ± S.E. of 5 animals for $^3$H-QNB binding. Values are mean of 3 separate of experiments for Scatchard analysis.

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

Na$^{+}$,K$^{+}$-ATPase activity exhibited a similar pattern of restoration as observed with AChE activity. Na$^{+}$,K$^{+}$-ATPase activity was restored by 70% in lead exposed and transplanted animal at both time periods while in iron-deficient lead exposed animals its activity was restored by 61% and 65% at 3 and 6 months post transplantation respectively (Fig. 28).

Monoamine oxidase activity a biochemical marker of the dopaminergic neurons was restored by 43% and 68% at 3 and 6 months respectively in lead exposed and transplanted rats. In the iron-deficient lead exposed rats MAO activity was restored by 31% and 60% at 3 and 6 months post transplantation respectively (Fig. 29).

Polyamine levels did not exhibited any significant increase in their levels at the end of 3 months post transplantation. Their levels, however, improved substantially and significantly at the end of 6 months. Putrescine levels increased by 66% in lead exposed and transplanted animals and by 52% in iron-deficient lead exposed animals.
Fig. 28: Effect of intra-cerebellar homotopic transplants in lead exposed and iron-deficient rats on the activity of Na\(^+\),K\(^+\)-ATPase, 3 and 6 months post transplantation. Values are mean (nmole 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).
Fig. 29: Effect of intra-cerebellar homotopic transplants in lead exposed and iron-deficient rats on the activity of monoamine oxidase, 3 and 6 months post transplantation. Values are mean (nmol 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).
at 6 months post transplantation (Fig. 30). Spermidine levels were restored by 77% in lead exposed and transplanted animals and by 68% in iron-deficient lead exposed animals at the end of 6 months (Fig. 31).

Lead levels were increased by two folds in the cerebellum of lead treated animals and by around 3 folds in the iron-deficient lead exposed animals. These levels had come down by around 60% at 3 months post transplantation and their levels were statistically non-significant when compared to the control levels at the end of 6 months (Table 14).

<table>
<thead>
<tr>
<th>Table 14</th>
<th>Lead levels in the cerebellar region of lead exposed and iron-deficient rats 3 and 6 months post transplantation.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Iron-sufficient (Control)</td>
</tr>
<tr>
<td>3 months</td>
<td>35 ± 7 (4)</td>
</tr>
<tr>
<td>6 months</td>
<td>42 ± 6 (4)</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, a = vs pair fed iron-sufficient (Control).

Morphological observations

Transplants were observed as clusters of cells within the deep cerebellar nuclei (Fig. 32). HRP labelling helped to identify individual neurons within the transplant. Neuronal cells with Golgi like staining were observed around the transplant. Transplanted cells integrated into the host tissue by extending their processes as observed in Fig. 33.
Fig. 30: Effect of intra-cerebellar homotopic transplants in lead exposed and iron-deficient rats on the levels of putrescine, 3 and 6 months post transplantation. Values are mean (nmoles/g wet weight of tissue) ± S.E. of 4 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. 31: Effect of intra-cerebellar homotopic transplants in lead exposed and iron-deficient rats on the levels of spermidine, 3 and 6 months post transplantation. Values are mean (nmoles wet weight of tissue) ± S.E. of 4 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. 32: Photomicrographs showing a dense cluster of transplanted cells within the deep cerebellar nuclei of lead exposed iron-deficient rats. ax150, bx400, cx500 (cresyl violet stained). The same transplants as seen under high power of microscope (d&e x 800).
Fig. 33: Golgi like staining of transplanted neuronal cells pre-labelled with horse radish peroxidase prior to transplantation. The brown precipitate as seen in the photographs is a reaction product of peroxidase with diaminobenzidine which was used as a chromogen. ax150, bx200, cx800 (diaminobenzidine stained).
posure to lead during development results in the impairment of learning and and deficits in locomotor functions. The exact mechanism(s) underlying havioural deficits is still obscure. Intra-hippocampal fetal transplants rich in gic neurons were able to partially restore neurochemical as well as be- ul deficits as observed in the previous study. In the present study, restorative 1 of fetal cerebellar transplants into the cerebellum of lead exposed animals ertaken.

tra cerebellar homotopic fetal transplants were able to restore motor deficits st control levels at the end of 6 months post transplantation. However, these ants failed to elicit any improvement in learning and memory. The observation ebellar transplants are unable to restore the learning and memory deficit in posed rats, lets credence to the fact that learning and memory involves the rgic system within the hippocampus, and that any treatment designed to e these deficits must address the issue of cholinergic dysfunction (Ridley and 1993).

he graft induced improvement of motor performance in lead treated rats s that: (1) the motor deficit as a result of lead exposure is a consequence of changes within the cerebellar region of the brains and (2) the degree of tion by cerebellar transplants is inversely related to the level of lead within the llum, and directly to the level of polyamines.

a rats the brain growth spurt, which is arbitrarily defined as the period at which wows most rapidly, takes place postnatally (Castiglioni et al, 1989). This is the hen polyamine levels are at their peak as they are required by the developing al and glial cells. Lead disrupts the polyamine pathway leading to a lower of these essential amines thereby causing subtle changes in the neuronal An important consequence of these changes may be loss of synaptic contacts en neurons and henceforth loss of motor coordination. As evident from the
estimated values obtained for lead and polyamine levels, motor activity of lead exposed and transplanted rats was improved only when lead levels had come down appreciably and polyamine levels had been restored significantly. Further, support to this hypothesis is lend by the data on the lead exposed iron-deficient animals, where lead levels are more and percent restoration is less when compared to the lead exposed and transplanted animals.

In the cerebellum, which acts as a modulator of fine motor reflexes, neuronal networks are organised in a point-to-point manner, indicating cell-cell interactions. In a series of experiments, Sotello and Alvarado-Mallart (1991) and Armengol et al (1989) demonstrated that when transplanted in the cerebellum of Purkinje cell deficient mutant mice, fetal cerebellar Purkinje neurons reconstruct the deficient cerebellar circuitry in a point to point manner. The restoration of lead induced biochemical and behavioural deficits by fetal cerebellar transplants especially motor performance and spontaneous locomotor activity at 6 months post transplantation, suggests partial restoration of the damaged cerebellar circuitry. However, the involvement of trophic influence of the graft, by way of releasing neuron promoting and nerve growth factors in enhancing the repair process, is also possible (Cohen, 1991).