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A Mechanism-based complementary screening approach for the amelioration and reversal of neurobehavioral teratogenicity

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Abstract

The identification of mechanisms and outcomes for neurobehavioral teratogenesis is critical to our ability to develop therapies to ameliorate or reverse the deleterious effects of exposure to developmental neurotoxicants. We established mechanistically-based complementary models for the study of cholinergic systems in the mouse and the chick, using both environmental neurotoxicants (chlorpyrifos, perfluoroalkyls) and drugs of abuse (heroin, nicotine, PCP). Behavioral evaluations were made using the Morris maze in the mouse, evaluating visuospatial memory related to hippocampal cholinergic systems, and imprinting in the chick, examining behavior dependent on cholinergic innervation of the IMHV. In both models we demonstrated the dependence of neurobehavioral deficits on impairment of cholinergic receptor-induced expression, and translocation of specific PKC isoforms. Understanding this mechanism, we were able to reverse both the synaptic and behavioral deficits with administration of neural progenitors. We discuss the prospects for clinical application of neural progenitor therapy, emphasizing protocols for reducing or eliminating immunologic rejection, as well as minimizing invasiveness of procedures through development of intravenous administration protocols.

Keywords

Chick; Induction of endogenous cells; Mouse; Neural progenitor transplantation; Neurobehavioral teratogenicity

1. Complementary neurobehavioral teratogenicity models

An inherent methodological obstacle in studies of the mechanisms of neurobehavioral teratogenicity is the problem of specificity; most neuroteratogens affect multiple regions and processes, resulting in plethora of behavioral defects. It is thus critical to develop exposure models that produce functional (behavioral) deficits linked to specific neurotransmitter and synaptic mechanisms. Accordingly, the principal approach taken in our studies has been to

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examine exposures to agents that may differ in the originating mechanism of action, but that nevertheless converge on common final pathways to result in parallel neurobehavioral disruption. As described below, we designed complementary models in both a mammalian species (mouse) and avian species (chick) that ultimately permitted identification of cell signaling deficits common to developmental exposure to a drug of abuse (heroin) as well as an environmental contaminant (the pesticide, chlorpyrifos), and then were able to use this to provide the groundwork for therapeutic reversal of the adverse effects.

1.1. The mouse model

In a mouse model, we focused on "region-specific behaviors" and their related synaptic alterations, in this case, the Morris and eight arm radial maze behaviors, which are entirely dependent on the integrity of septohippocampal ACh innervation [3,24,31,35–37,50]. We employed heroin and phenobarbital as archetypal teratogens but also examined the effects of chlorpyrifos, nicotine, ethanol and PCP. Here, we will focus on heroin, phenobarbital and chlorpyrifos. These substances obviously differ in their chemical class and basic mechanisms of action, but all three nevertheless converge on the development of hippocampal cholinergic innervation [32,33,55,57]. We previously established that prenatal treatment of HS/Ibg heterogeneous stock mice [28] with these teratogens evoked persistent presynaptic hyperactivity, evidenced by increases in the concentration of choline transporter sites [44,56] and by a tonic increase in ACh release [1]. Significantly, whereas we expected to see compensatory postsynaptic receptor downregulation and desensitization, we instead found *upregulation* of muscarinic receptors and of the mechanisms coupling the receptors to cellular function (G-protein linkages and carbachol-induced IP formation) [46,55,56]. This implied that there is an underlying defect in ACh signaling, producing upregulation of both presynaptic and postsynaptic elements of ACh function that nevertheless failed to restore septohippocampal behavioral performance to normal. Subsequently, we discovered that the uncoupling of ACh synaptic activity from its behavioral functions resided in a specific defect, centered around PKC. With each of the teratogens, there was a specific defect centered around the function of PKC, a total abolition of cholinergic receptor-induced activation and translocation to the membrane for the γ and β isoforms [1,4,20,46,48,55–57,69]. Importantly, no such defect was seen for PKC α , an isoform known **not** to participate in septohippocampal ACh-related behavioral function. The specificity of this defect was even more evident by the high correlation between impaired PKC function and behavioral performance within individual animals [57]. In turn, as described later, identification of this mechanism gave us important information for designing therapies that could reverse the neurobehavioral and synaptic deficits.

1.2. The chick model

Having established a region-specific mechanism-based model in rodents, we then designed a complementary chick model according to the same principle. In this case, the behavioral end point was filial imprinting, with the same type of cholinergic involvement converging on the intermediate hyperstriatum ventrale (IMHV), a region in the chick that plays the same role as the hippocampus in mammals. Although rodent models are more similar to humans, there are distinct advantages to the chick model that complement mammalian studies. The rodent model has inherent methodological shortcomings from confounding indirect variables related to maternal effects, [47,52] such as maternal care and mother-offspring interaction [2,15,30,45], and disruption of maternal endocrine status, all which affect behavioral outcomes. Obviously, the avian model avoids these confounds, since the teratogen is injected into the egg, so that the embryo is directly exposed to a defined concentration; the exposure thus resembles the zebrafish model [23], in which eggs are placed in a constant concentration in the medium (water) and are thus exposed to a uniform level of teratogen. Similarly, although the rodent model exhibits a "litter effect" [54] this limitation is absent in the chick model; every individual offspring represents an independent sample. Finally, chick eggs are cheap, abundant, and easy

to maintain in large numbers, and thus are more suitable for higher-throughput screening than mammalian models. Ultimately, we believe it most advantageous to work simultaneously with both chicks and rodents to reap the benefits of each model.

Accordingly, we designed studies to examine the effects of prehatch exposure of the chick embryo to the “cholinergic teratogens” chlorpyrifos, heroin and nicotine, exactly parallel to our work in the mouse model. Each of the agents induced deficits in imprinting behavior, and just as in the rodent model, targeted the function of PKC γ and PKC β ; the effects were most notable in the left IMHV, the anatomical site related to ACh input that regulates imprinting behavior and thus parallel to the hippocampus in the mouse model. As in the mouse model, PKC α , which is not involved in these behaviors, was not affected by the prehatch exposure. Further studies established a protocol for the assessment of ACh receptor-induced translocation/activation of PKC γ , and as in the mouse, the teratogen-exposed chicks showed abolition of this process [20].

These findings demonstrated the effectiveness of the chick model for neurobehavioral teratogenicity. Subsequently, we applied the model to compounds for which neurotoxicant effects were suspected, but not yet proven, such as perfluoroalkyl chemicals, which are of increasing concern due to their environmental ubiquity and subsequent accumulation in the mammalian fetus. Chicken eggs were injected with varying doses of perfluorooctanoic acid (PFOA) or perfluorooctane sulfonate (PFOS) prior to incubation. Defects were observed in hatching, increased incidence of splayed legs and, for PFOA, interference with the appropriate development of yellow plumage [64]. More recent studies indicate deficits in imprinting behavior after prehatch exposure to each chemical independently (Unpublished observations), so that these compounds, too, may turn out to compromise the same ACh pathways as do some of the already identified developmental neurotoxicants. Again, the point here is that the chick model facilitated the rapid screening of a series of these compounds, enabling dose-response determinations, a focus on critical periods of exposure, and likely endpoints to be studied.

2. Reversal of neurobehavioral teratogenicity

Because we identified septohippocampal (mouse) and IMHV (chick) ACh pathways, and PKC γ function, as the mechanisms underlying the neurobehavioral teratogenesis of a variety of otherwise unrelated compounds, we were next poised to use this information to design therapies to reverse the behavioral deficits and the corresponding synaptic alterations.

2.1. Reversal with 6-hydroxydopamine (6-OHDA): Manipulations of the regulating neural pathways

The A10-septal dopaminergic pathways provide negative modulation of septohippocampal ACh function through GABAergic mediation. In mice offspring exposed prenatally to phenobarbital, we lesioned the A10 cells using localized injection of the specific neurotoxin, 6-OHDA. The treated offspring showed a corresponding improvement in their eight-arm maze learning [65,66,68].

2.2. Reversal with nicotine therapy

In the next set of studies, we focused on restoring function by applying direct ACh agonists. Mice exposed to phenobarbital prenatally received chronic treatment with nicotine via osmotic minipumps. The treatment totally reversed the prenatal phenobarbital-induced deficits in Morris maze behavior and restored cholinergic receptor-induced translocation/activation of PKC γ [4]. We then went on to show a similar reversal in animals exposed to chlorpyrifos using injections of nicotine delivered just before each day's test in the Morris maze (Billuauer et al.,

manuscript in preparation). The efficacy of acute nicotine therapy has been applied similarly by others to offset the neurobehavioral teratogenicity of lead [72].

2.3. Reversal with neural grafting of differentiated cholinergic neurons

Neural grafting in this paper refers to grafting of differentiated, embryonic cells [7], as opposed to *transplantation of neural progenitors*, which involves undifferentiated stem cells that nevertheless are already committed to become nerve cells.

Mice were exposed to heroin prenatally were grafted on age 35 days with normal embryonic cholinergic neurons. Just like nicotine administration, this therapy totally reversed the prenatal heroin-induced behavioral deficits and restored the cholinergic receptor-induced translocation/activation of PKC γ [46,55,57,67]. As will be discussed below, neural grafting is not clinically feasible and our focus then shifted to therapy with neural progenitor transplantation.

2.4. Reversal with neural progenitors transplantation

2.4.1. Reversal with neural progenitors in a mouse model—Using similar methodology as in neural grafting, we transplanted neural progenitors derived from the cortex of normal newborn mice to the hippocampal area of mice offspring which were exposed to heroin prenatally. Again, similar to the neural grafting therapy, transplantation of neural progenitors totally reversed the deficits induced by the teratogen in Morris maze behavior and restored ACh receptor-induced translocation/activation of PKC γ and β [21]. The findings were replicated with the chlorpyrifos exposure model (Billuauer et al., manuscript in preparation).

The clinical feasibility of neural progenitor therapy would be greatly facilitated by an ability to produce an immortalized tissue culture as a source of transplanted cells, one that contains cells that will differentiate into the desired neuronal phenotypes and that can be engineered to contain the appropriate factors to support their therapeutic actions. To this end, we transplanted neural progenitors derived from murine ES cell line culture (provided by Michel Revel [71]) into the hippocampal area of 35 day old mice which had been exposed to heroin prenatally. Before transplantation, a large number of nestin expressing cells could be identified in the culture, suggesting that they were indeed neural progenitors. After withdrawal of growth factors, the cells differentiated into neurons (neurofilament as indicated by Class III β -tubulin expression) astrocytes (GFAP) and oligodendrocytes (O₄). Preliminary observations suggest that the survival of the murine ES cell line cells after transplantation may be better than for neural progenitor cells derived from the newborn cortex. Functionally, the ES line-derived neural progenitors totally reversed functional deficits as tested in the Morris maze (Kazma et al., manuscript in preparation).

Studies by other groups led to similar conclusions regarding the efficacy of immortalized cultures. Neural progenitors derived from these cultures migrated to damaged areas of the mouse brain and then differentiated at the sites of impairment [8]. These types of cultures may also reach a higher proportion of cells differentiating into neurons as compared to other techniques, such as neurosphere cultures [10]. Indeed, the cells survived for at least one year [49] and reversed Parkinson's-like behavior in a mouse model [38]. Studies applying murine culture therapy in mouse models of traumatic brain injury and Huntington's disease mice, also showed alleviation of the behavioral deficits [53] [49,70].

2.4.2. Reversal with neural progenitors in a chick model—Based on the efficacy of the mouse model, we expanded the model to chicks [11]. First, we developed a method to derive neural progenitors from the chick embryo, establishing embryonic day 10 as the optimal point; not surprisingly, derivation of cells from more developed embryos (embryonic days 12,13) resulted in less neuronal and more glial differentiation. With derivation on embryonic

day 10, almost all the culture cells were nestin positive, suggesting that they were indeed neural progenitors. After removal of the growth factors to allow differentiation to proceed, all major lineages of the nervous system could be found, including neurons (Beta III tubulin-positive, 50% of the total number of cells), astrocytes (GFAP-positive, 30%), and oligodendrocytes (O₄-positive, 20%). We then labeled the neural progenitors with Dil cell tracer [16] and transplanted them to newly hatched chicks which had undergone prehatch chlorpyrifos exposure. The transplanted cells survived in the damaged host brain as attested to by the presence of the Dil-labeled cells on posthatch day 14. At the present preliminary stage of the study, the transplanted cells were detected in the host brain. The obvious next step is identifying the fate of those cells, whether they differentiate to neural or glial cells, and more specifically, which type of each.

2.4.3. Mechanism for the therapeutic action of neural progenitors—We conducted an extensive immunocytochemistry study to ascertain the survival and the differentiation of the transplanted cells in the host brain. Surprisingly, and despite the great therapeutic efficacy, the number of the surviving transplanted cells was relatively small. Most of the surviving cells (21.6%) differentiated into astrocytes as shown by GFAP labeling, whereas no Beta III tubulin-positive filaments or NeuN-positive nuclei in transplanted cells could be found, which indicates an absence of transplant-derived neurons [5,21]. This led us to the hypothesis that, whereas a portion of the transplanted progenitors may replace neurons and restore damaged circuits directly [14,51], the transplanted neural progenitors also induce a change in the host brain, possibly involving the release of cytokines, which in turn causes an increase in endogenous proliferation of cells that then produce the majority of the repair. Indeed, in subsequent studies, we found that transplantation of neural progenitors enhanced the production of neural precursors in the damaged brain [5]. Our conclusion is bolstered by three sets of already-known facts: **a.** Neural progenitors express cytokines [25,38,63], **b.** Cytokines enhance neurogenesis in neurogenic regions of the brain [13,59] and **c.** Endogenous hippocampal cell proliferation positively correlates with cognitive function [12,22]. Confirming the induction of neural progenitor hypothesis in the chick model, and studying the origin and differentiation of the induced endogenous precursors, are important areas for future investigations.

2.4.4. Clinical prospects for neural progenitor therapy—The prospect for application of neural progenitor therapy in humans should be viewed in light of what we already know from studies of neural grafting. The potential for grafting differentiated embryonic neural cells or tissues as a therapy to reverse functional (behavioral) deficits was first brought to the attention of the scientific community with the alleviation of Parkinson's-like syndromes in rats, in that case involving embryonic cells expressing a dopamine phenotype [7,41]. This approach was then extended to a variety of defects [6]. The initial obstacles for similar studies in primates were eventually overcome [9] and by the late 1980s several dramatic clinical reports reinforced the potential utility of this approach [26,27]. Clinical trials were initiated under the assumption that neural grafting would become the therapy of choice for Parkinson's disease in specific cases [17], but alarmingly, follow-up studies on the treated patients showed the emergence of crippling dyskinesias [18,34]. Consequently, at least for now, therapies using these types of neural grafts are clinically impractical.

The subsequent development of therapies using neural progenitors likewise appears to be very successful in animal models and has contributed to our understanding of the mechanisms by which neuroteratogens produce their functional defects [5,19]. Nevertheless, neural progenitor therapy has yet to cross into the realm of clinical applicability and the absence of progression to clinical trials after many years of research remains a concern. However, as detailed in the next sections, this approach gives promise both as a tool for development of novel therapies, for the prospect of deriving progenitors from adult cells, and for noninvasive administration, factors that are not shared by the other approaches.

2.4.5. Prospects for neural progenitors as a tool for developing therapies to reverse neurobehavioral teratogenicity, for derivation from adult cells, and for noninvasive administration—

Despite the potential problems in realizing the complete clinical potential of neural progenitor therapy, this approach already provides a useful tool to identify in animal models how reversal may be achieved, in turn leading to the development of more facile therapies. For example, as discussed above, neural progenitor transplantation may allow us to identify which cytokines are actually providing therapeutic effects, such as induction of endogenous neural precursors. In order to screen for the relevant cytokines, which may themselves be considered the therapeutic agent, inducing endogenous proliferation of neural precursors and consequently reversing the defect, neural progenitor transplantation provides a particularly suitable probe. Once this probe identifies the cytokines, direct administration of the specific cytokine, or cocktail of cytokines, may then become a targeted therapy for the reversal of neurobehavioral damage, bypassing the use of neural progenitors, nearing closer to clinical feasibility.

The development of neural progenitor sources from immortalized or at least long-lasting cell cultures has obvious advantages over earlier approaches using neurospheres derived from the newborn cortex. The next aim would be for a patient's own tissue to be the source of the progenitor cells. The most readily available source of adult neural progenitors is from the nervous system, mainly neurogenic tissues such as the SVZ [40,61], hippocampus [39,40,61] or olfactory bulb [62], but also from non-neurogenic areas such as the septum and, striatum [40], spinal cord [61], optic nerve and neocortex [39]. While obtaining the requisite brain samples may provide some difficulty, additional protocols have been developed to reprogram somatic cells derived from human fibroblasts to become stem cells; however, this requires procedures such as transfection of genes into the non-pluripotent cells, which is labor-intensive [58]. Simpler procedures which avoid the complexity of transfection have been developed, such as obtaining stem cells from hair follicles, an area which is naturally self-renewing even in adults. Toward this end Valenzuela et al. derived cells from canine skin which formed neurospheres in culture. However, these skin-derived neuroprecursor cells have yet to be explored for their ability to survive and function after transplantation [60]. Recently, of great significance, adult stem cells were derived from human olfactory mucosa. These cells were propagated as neurosphere culture, similar to other neural stem cells. They differentiated along the dopaminergic lineage as attested to by tyrosine hydroxylase expression and by the release of dopamine. The neural progenitors were transplanted into the brain of hemi-parkinsonian rats lesioned unilaterally in the striatum by stereotaxic injection of 6-hydroxydopamine, which resulted in improvement of their Parkinson's-related behavioral deficiencies as assessed by evaluation of rotational behavior [29]. Accordingly, this represents a promising approach.

Once having optimized the source of neural progenitors and procedures for deriving cells from that source, we need to design protocols to permit the least-invasive administration of the cells into the deficient host, preferably involving injection into the bloodstream or other peripheral sites rather than into the brain itself. Neural progenitors derived from the neonatal cortex and periventricular region of the forebrain ventricles of adult mice have been administered intravenously to EAE mice (multiple sclerosis model). Before transplantation a procedure were applied to force the opening of the blood-brain barrier of the host by inducing inflammatory-like conditions. The transplanted cells survived and could be detected later within damaged areas of the CNS, producing significant histological and functional (motor) improvement [42,43]. Again, this indicates a positive long-term prospect for the application of these techniques to offset neurobehavioral deficits in humans.

3. Conclusions

In the field of neurobehavioral teratogenicity, it is not sufficient to identify agents that disrupt brain development and behavioral performance. Rather, it is equally critical to design therapies that can offset or reverse the effects of developmental damage. To that end, it is necessary to develop models that permit mechanistic characterization of functional synaptic defects, to identify the connection between deficits at the cellular level and behavioral outcomes, and then to develop therapeutic strategies that target these mechanisms. We developed two complementary models in the mouse and the chick, showing parallel cholinergic mechanisms and behavioral outcomes that represent common final pathways for a variety of otherwise unrelated neurotoxicants. Both models focused on cholinergically-mediated behaviors with defined anatomical localizations: Morris and radial mazes in the mouse, related to septohippocampal cholinergic input; and imprinting performance in the chick, related to parallel circuitry located in the IMHV. We then showed how both these models provide useful information: the chick model enables higher-throughput, rapid screening of multiple compounds without maternal or litter confounds, whereas the mouse model more closely resembles humans and is thus of greater relevance regarding potential clinical use. But most importantly, both models enabled the testing of potential therapeutic strategies designed to reverse the effects of neuroteratogen exposure; neural progenitor therapy represents the most promising avenue for future pursuit.

Neural progenitor therapy, if made feasible in humans, offers significant clinical potential – even more so if application protocols are made simple and minimally invasive. Recent and anticipated studies are progressing toward a readily available source of neural progenitor cells which elicit minimal immune rejection, perhaps even deriving them from the patient's own somatic tissue, and permitting administration through the peripheral vasculature. Clearly, this is an area of tremendous potential and complementary models will play a large role in our progress toward these goals.

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Abbreviations

6-OHDA	6-hydroxydopamine
ACh	acetylcholine
EAE	experimental autoimmune encephalomyelitis
GABA	gamma-aminobutyric acid
GFAP	glial fibrillary acidic protein
HC3	hemicholinium-3
IMHV	intermediate hyperstriatum ventrale
PFOA	perfluorooctanoic acid
PFOS	perfluorooctane sulfonate
PKC	protein kinase C.

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