Functional changes after prenatal opiate exposure related to opiate receptors’ regulated alterations in cholinergic innervation

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Abstract

Opioid drugs act primarily on the opiate receptors; they also exert their effect on other innervations resulting in non-opioidergic behavioural deficits. Similarly, opioid neurobehavioural teratogenicity is attested in numerous behaviours and neural processes which hinder the research on the mechanisms involved. Therefore, in order to be able to ascertain the mechanism we have established an animal (mouse) model for the teratogenicity induced by opioid abuse, which focused on behaviours related to specific brain area and innervation. Diacetylmorphine (heroin) and not morphine was applied because heroin exerts a unique action, distinguished from that of morphine. Pregnant mice were exposed to heroin (10 mg/kg per day) and the offspring were tested for behavioural deficits and biochemical alterations related to the septohippocampal cholinergic innervation. Some studies employing the chick embryo were concomitantly added as a control for the confounding indirect variables. Prenatal exposure to heroin in mice induced global hyperactivation both pre- and post-synaptic along the septohippocampal cholinergic innervation, including basal protein kinase C (PKC) activity accompanied by a desensitization of PKC activity in response to cholinergic agonist. Functionally, the heroin-exposed offspring displayed deficits in hippocampus-related behaviours, suggesting deficits in the net output of the septohippocampal cholinergic innervation. Grafting of cholinergic cells to the impaired hippocampus reversed both pre- and post-synaptic hyperactivity, resensitized PKC activity, and restored the associated behaviours to normality. Consistently, correlation studies point to the relative importance of PKC to the behavioural deficits. The chick model, which dealt with imprinting related to a different brain region, confirmed that the effect of heroin is direct. Taken together with studies by others on the effect of prenatal exposure to opioids on the opioidergic innervation and with what is known on the opioid regulation of the cholinergic innervation, it appears that heroin exerts its neuroteratogenicity by inducing alterations in the opioidergic innervation, which by means of its regulatory action, attenuates the functional output of the cholinergic innervation. In our model, there was hyperactivity mostly of the post-synaptic components of the cholinergic innervation. However, the net cholinergic output is decreased because PKC is desensitized to the effect of the cholinergic agonist, and this is further evidenced by the extensive deficits in the related behaviours.

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Introduction and overview

Among the opioidergic drugs, heroin is the major drug of abuse that possesses great addictive potential and it appears to maintain its notoriety despite transient competition for this title by other types of drugs. A probable reason is that, as an opiate, heroin directly targets the sites that reinforce the addictive behaviour, the opioid innervation. Although other addictive drugs, such as ethanol, also interact with the opioidergic innervation (Charness, 1989; Town et al., 2000), the interaction appears not to be as direct as in the case of heroin.

Heroin abuse is prevalent, illustrated in the increase of abuse in the USA; based on the 1991–1995 National
Household Survey on Drug Abuse, heroin abuse has more than tripled in the USA between 1992 and 1996 (Epstein and Grober, 1998). Heroin abuse has become more common among the young population; for example, from 1993 to 1995, 88% of heroin users were aged between 12 and 25 yr. Consequently heroin users are more frequently of childbearing age. In fact, it has long been established that at least one delivery out of 1000 in the USA is a ‘heroin baby’ (Carr, 1975), this accounts for a total of over 300,000 ‘heroin baby’ births in a period of two decades (Zagon et al., 1984).

The central issue dealt with in this article is the animal model for the neurobehavioural teratogenicity induced by narcotic addiction. The major relevant substance of abuse among the opioid drugs is diacetylmorphine (heroin) and therefore studies published on related opioids, methadone, 1-alpha-acetylmethadol (LAAM), or buprenorphine will be mentioned only briefly here. While heroin is the principal neurobehavioural teratogen, almost all research on an animal model for heroin neuroteratogenicity has employed morphine, assuming that the effect of the two drugs is essentially identical since heroin readily converts to morphine upon entering the body. We will argue below for the employment of heroin in models of neuroteratogenicity due to its unique action not shared by morphine. Since opiates are obviously opioidergic drugs, it is not surprising that most biochemical studies on their neuroteratogenicity focused on endogenous opiates and opiate receptors as end-points. Our model is devoted to non-opioidergic mechanisms of heroin neuroteratogenicity. It appears that heroin acts on the non-opioidergic innervation via the regulating opioidergic innervation or even directly (Khananshvili and Sarne, 1992; Lapchak et al., 1989; Sarne et al., 1991).

The task of elucidating the mechanism of heroin-induced behavioural birth defects is made difficult because it appears that opioids, like many other neuroteratogens, act diffusely in the brain and affect numerous brain regions and processes resulting in multiple, seemingly unrelated behavioural deficits. We have attempted to counter this methodological hindrance by focusing the research on ‘region-specific behaviours’. That is, the study of early heroin-induced alterations in defined brain areas and on their related behavioural deficits; specifically, early heroin-induced alterations in septohippocampal cholinergic innervation and in hippocampus-related behaviours (Abu-Rumi et al., 1996; Shahak et al., 2003; Steingart et al., 1998, 2000b; Yanai et al., 1992b). Obviously, the possible role of other brain regions and innervations in the behavioural deficits was not overlooked (Slotkin et al., 2001). Our animal model for heroin neuroteratogenicity employed mice due to their obvious advantage as mammals. As a control for the possibility of confounding indirect variables, such as maternal physiology or mother–offspring interaction, several studies on the chick model have been recently added.

The ascertaining of deficits in behaviours related to specific innervation in mice exposed prenatally to heroin, and the extensive investigation of the pre- and post-synaptic components of this innervation, which are correlated with the behavioural deficits, enables the reversal of the behavioural deficits and the neural alterations with neural grafting. In turn, neural grafting can be used as a probe for understanding the mechanism of the behavioural deficits, and thus facilitates an understanding for studies on the reversal of heroin-induced neurobehavioural teratogenicity using other techniques.

This study was conducted in accordance with the National Institute of Health Guidelines for the Care and Use of Animals in Research and under protocols approved by the Animal Care and Use Committee of the Hebrew University of Jerusalem.

The generality of opioid action on the brain

Our model on heroin neurobehavioural teratogenicity was designed to ascertain the mechanism by which the drug, when given prenatally, exerts its effect on the development of behaviour. In this model pregnant mice were exposed to heroin (a single injection of 10 mg/kg) on gestation days (GD) 9–18 and the offspring were tested at adulthood for deficits in behaviours related to the hippocampus and concomitant alterations in the septohippocampal cholinergic innervation. The design of the study focused on behaviours related to specific innervation, and this was necessitated by the fact that opioids, like many other neuroteratogens, affect numerous brain regions and processes resulting in multiple behavioural deficits. Opioids primarily target the opioidergic innervation and consequently the major changes demonstrated after opioid exposure in adulthood are in the biochemistry of this innervation and in the resulting opioidergic behaviours as, for example, is the case in analgesia (Hayes and Vogelsang, 1991) or the decrease in the immunoreactivity of adenylate cyclase among heroin addicts (Shichinohe et al., 2001). Additionally, there are apparent general effects of heroin where the role of the opioid receptors is only in the regulation of other innervations or it may even be that they are entirely uninvolved (Khananshvili and Sarne, 1992; Sarne et al., 1991). Possible examples of the general
effects are the impairment of the planning functions of the prefrontal cortex as assessed with various standard performance tests. This damage can be explained by cumulative neuronal damages of prefrontal cortex and ventral tegmental area (VTA) dopamine neurons (Brun et al., 2001). Another example is the alteration of the immune response by opiates (Mellon and Bayer, 1998; Stefano et al., 1996).

**The generality of the neurobehavioural teratogenicity of opioids**

**Human studies**

When consumed during pregnancy in humans and animals, opiates, including heroin, readily cross the placenta and the blood–brain barrier. As in exposure at adulthood they are distributed all over the fetal brain and are capable of affecting numerous innervations in addition to their affect on the opioidergic innervation. Again similar to exposure at adulthood, the effect on the non-opioidergic innervations can be either indirect via opioidergic regulation or direct on the non-opioidergic innervations as an expression of their action as general neuroteratogens. Behaviourally, it follows that opioid teratogenicity, beyond the changes in the opioidergic function, covers a broad spectrum of seemingly unrelated behaviours that are attributed to various brain regions and neurotransmitter innervations. An indication in humans for the direct changes in the opioidergic function is the neonatal withdrawal syndrome of babies born to narcotic-addicted mothers. In a less specific action, prenatal opioid exposure caused an inability to concentrate, apathy, lessened physical activity and reduced visual ability. Learned responses are impaired along with mental and physical performance (Calman and Strang, 1962; Doberczak et al., 1991). Between the ages of 5 and 12 yr, children born to heroin-dependent mothers revealed an increased prevalence of attention deficit hyperactivity disorder (ADHD) as well as other behavioural disorders; many of which were present in the heroin-exposed children even if they were adopted by drug-free parents (Ornoy et al., 2001).

Identifying specific heroin-induced neurobehavioural defects in human development has proved to be elusive: confounding factors prevail in the addict population, notably the co-abuse of other drugs, poor nutrition and infectious diseases (Ornoy et al., 1996, 2001). Furthermore, the level of opiate consumption is usually unknown and self-reportage is notoriously unreliable (Ornoy et al., 1996, 2001). The use of animal models, including our model of prenatal opiate exposure, obviates these confounding factors. These models revealed a multitude of neurobehavioural alterations related to numerous brain regions and innervations.

**Animal models**

On the cellular level, opioid-exposed rat offspring had alterations in brain cell numbers, synaptic development mainly the catecholaminergic, brain RNA and protein as well as changes in polyamine metabolism (Vathy and Katay, 1992; Vathy et al., 1994; Zagon et al., 1984). General behavioural deficits of prenatal heroin exposure in rats include hyperactivity as assessed in the activity cage, open field and activity wheel (Lasky et al., 1977). Other deficits were in tasks requiring learning and memory (Braida et al., 1994; Canli et al., 1990; Gallagher et al., 1985; Slamberova et al., 2001; Spain and Newsom, 1991), alterations in social and sexual behaviour (Hol et al., 1996; Niesink et al., 1996, 1999; Vathy, 1999), and changes in responsiveness to stress and stimulants (Castellano and Ammassari-Teule, 1984; Vathy, 1999).

**The region-specific model: targeting the septohippocampal cholinergic innervation – results and discussion**

**Methodological issues**

Due to the generality of the heroin effects as shown above, our animal model on the mechanism of behavioural deficits induced by prenatal heroin exposure, was designed to investigate behavioural deficits which are specific to certain brain regions and neurochemical processes (Abu-Roumi et al., 1996; Shahak et al., 2003; Steingart et al., 1998, 2000a,b; Yanai et al., 1992b). The description of our model will emphasize several methodological issues with general relevance to neurobehavioural teratology.

**Strain**

We used heterogeneous stock (HS/lbg) mice, a population which was obtained by the crossing of eight inbred strains and then maintained intentionally heterogeneous (McClearn et al., 1970). Heterogeneous stocks appear to be advantageous as a tool in neuroteratogenic models as compared to inbred strains since (a) the dam can maintain pregnancy even under the exposure to insults, and (b) the heterogeneous population possesses a wide genetic repertoire, so that drawing a biased conclusion due to the possible genetic uniqueness, as often occurs in research with inbred strains, is avoided.
Drug

Most research on the animal model for the neurobehavioural teratology of heroin intake employ morphine to represent heroin (a few studies which employed heroin were also published; Lasky et al., 1977; Zhu and Stadlin, 2000). This is because heroin, upon entering the body, metabolizes to 6-acetylmorphine and then further de-acetylates to yield morphine (Inturrisi et al., 1983). The use of heroin in our model is justified since accumulating data continuously emphasizes the uniqueness of the heroin action compared to that of morphine. In fact, drug addicts are able to distinguish between morphine and heroin (Martin and Fraser, 1961). The very rapid uptake of heroin to the brain compared to morphine may have not only a quantitative, but also a qualitative significance (Oldendorf et al., 1972). Furthermore, recent work clearly demonstrates a different mode of action for heroin compared to morphine in that heroin, similar to morphine-6β-glucuronide (M6G), acts through a unique receptor which is not sensitive to morphine (Pasternak, 2001; Rossi et al., 1996). This notion was supported with studies representing several lines of evidence. For example, knockout mice containing disruptions of coding exons of MOR-1 (Schuller et al., 1999) were insensitive to morphine but were still responsive to heroin analgesia, and related studies demonstrated a deferential response to the two opioids across mice strains (Rady et al., 1991). Consistently, various manipulations differentially affect the function of the two drugs (Brown et al., 1997; Rossi et al., 1996). Whether or not heroin indeed, acts uniquely on a slice variant of the μ-receptor, it is clear that some of the heroin action is different than that of morphine and that the use of heroin in our model on narcotic neurobehavioural teratogenicity was warranted.

Mode of drug administration

Prenatal exposure was carried out by single daily subcutaneous injections (10 mg/kg). This was done from necessity because multiple daily injections which best simulate the drug addiction situation would be too stressful to the mouse, potentially inducing confounding indirect effects that would result in an unacceptable rate of fetal death. Administration of neuroteratogens, including opioids, via osmotic minipumps was carried out by several groups to minimize the indirect effects of the neuroteratogens and to moderate the peaks and valleys of drug levels (Ernest et al., 1998; Robinson et al., 1997; Slotkin, 1998). While the advantages of this procedure are obvious, we still chose to deliver the drug by injection which better simulates the situation in the addicted mother. In the case of heroin, the peaks and valleys of the drug levels are an essential part of the heroin addiction reality.

Period of prenatal exposure

Heroin was administered on GD 9–18, although administration throughout the pregnancy would be preferred as it better simulates the human situation. This schedule was necessitated by the results of our preliminary studies which demonstrated that the administration prior to GD 9, under the conditions of our model, resulted in a high level of fetal resorption. Similarly, removal of the drug on GD 18 prevents postnatal withdrawal and excessive fetal death (J. Yanai, unpublished observations). Fostering of control and heroin-exposed offspring by control dams was carried out since alterations in maternal care and mother–pup interactions have been observed with neuroteratogens, ethanol to name one prominent example (Kelly et al., 2000). However, recent studies demonstrated that under our experimental conditions the heroin-exposed mothers exhibited normal maternal care (Shahak et al., 2003).

Behavioural deficits

Due to the diffused nature of the heroin effect in the brain, as discussed above and in previous publications (Yanai, 1984), our model employed behaviours known to be primarily related to specific brain regions and neurochemical processes. We chose the behaviours in the eight-arm and Morris mazes which are known as indicators of spatial memory and discrimination (Morris, 1984; Olton and Samuelson, 1976). The eight-arm maze is built in a radial form and the ability of the mouse to find a drop of water in each arm while avoiding entries to previously visited arms assesses its short-term memory and spatial discrimination. The same abilities are assessed in the Morris maze where the mouse is placed in a water pool and has to find a submerged platform in order to get out of the water. These behaviours are related primarily to the septo-hippocampal cholinergic innervation which appears to be particularly relevant due to its regulatory interaction with the opioidergic innervation. Hippocampal specificity of the eight-arm maze behaviour has been demonstrated by electrophysiological studies (Olton et al., 1978a,b), lesions (Becker et al., 1980; Olton and Papas, 1979) and cholinergic pharmacological manipulations (Low et al., 1982). Similar evidence related to the Morris maze qualifies this behaviour as another standard hippocampus-related performance (Morris,
Functional changes and alterations in cholinergic innervation

Neurochemical alterations

A systematic study was conducted to ascertain the components within the hippocampal cholinergic transmission cascade that are the potential mechanisms of the heroin behavioural teratogenicity. In the study of the presynaptic components, the activities of the acetylcholine (ACh) synthesizing enzyme, choline acetyltransferase (ChAT) and the ACh-hydrolysing enzyme, acetylcholinesterase (AChE) were not studied in animals prenatally exposed to heroin. However, parallel studies on a related neuroteratogen, pheno-barbital, showed no alterations in either enzyme after prenatal exposure (Kleinberger and Yanai, 1985; Rogel-Fuchs et al., 1992). Much can be inferred from pheno-barbital studies to heroin studies since both neuroteratogens act directly (phenobarbital) or indirectly (heroin) on the development of the cholinergic innervation. In retrospect, this lack of the neuroteratogen effect should not be surprising because both enzymes are not rate-limiting. On the other hand, the study of choline transporter sites appeared to represent the most important component since its activity is rate-limiting in ACh synthesis and is responsive to the rate of neural firing (Kristofikova et al., 1995; Simon et al., 1976; Zahalka et al., 1993). Offspring exposed to heroin, prenatally, showed an increase in the number of high-affinity choline transporter sites assessed with the specific radioligand, hemicholinium-3 (HC-3) (Steingart et al., 1998, 2000b) suggesting the occurrence of presynaptic hyperactivity. A subsequent and more detailed study was conducted which, using HC-3 autoradiography, pinpoints the alterations to the behaviourally relevant subregion, hippocampal CA1 (Yanai et al., 2001). ACh release, assessed indirectly by measuring inositol phosphate (IP) formation in the presence of K⁺, was also increased after prenatal exposure to heroin (Abu-Roumi et al., 1996), which further supports the suggestion of presynaptic hyperactivity.

The evaluation of post-synaptic components was carried out on the expression/function of the muscarinic receptors as well as the cell signalling cascades controlled by the receptors as indicators for alterations in post-synaptic signalling. Based on what is known from studies on adults, it was expected that postsynaptic down-regulation would occur as an adaptive response to presynaptic up-regulation. Unexpectedly, however, mice exposed to heroin prenatally had an increase in the number (Bmax) of the muscarinic receptors while the affinity constant (Kd) remained unchanged (Yanai et al., 1992a). Out of the known muscarinic receptor subtypes M1 appears most pertinent to the present study because it is the predominant muscarinic subtype in the hippocampus (Kellar et al., 1985) and is most related to the behaviours studied in the present model. Consistent with the study on the general population of hippocampal muscarinic receptors which employed quinuclidinyl benzilate (QNB), pirenzepine binding revealed an increase of M1 receptors among the heroin-exposed offspring (Steingart et al., 2000a; Yanai et al., 1992a).

Further studies were carried out on the initial steps linking the receptors to cellular function, G-protein (GP) activation and carbachol-induced IP formation (Abu-Roumi et al., 1996; Steingart et al., 1998, 2000a). GPs provide the link from receptors to the second messenger (Berridge, 1985), in the case of M1, to IP. In our study, early heroin-treated offspring showed higher activation of hippocampal GP than control offspring as indicated by IP formation in response to NaF/AlCl3 (Abu-Roumi et al., 1996). NaF stimulates phosphoinositide hydrolysis by mimicking the action of the γ-phosphate group of GTP, thus inducing dissociation of the α subunit from the βγ subunits of the guanine nucleotide-binding protein Gq which is linked to phospholipase C (Smrcka et al., 1991). However the hyperactivation observed in GP is non-specific because it represents alterations in the general GP pool which is utilized by various neurotransmitter systems, and because it is unable to differentiate between GP subtypes. Consequently, we quantitated the amounts the GP subtypes, Gq, Gi and Go in control
and heroin-treated offspring by immunoblotting. An increase from the control level was found in Gq and Gi subtypes in heroin-treated offspring, while Go remained unchanged (Steingart et al., 1998). The change in Gq is particularly important since this subtype is activated by M1 receptors, stimulates IP formation and activates PKC. Similar to the case of GP, direct quantitative assessment of IP, the second messenger associated with M1 (as well as with M3 and M5) is non-specific because it represents alterations in the general IP pool which is utilized by various neurotransmitter systems. However, evaluation of IP formation after incubation of hippocampal slices with the cholinergic agonist, carbachol, showed an increase in IP formation in the treated group as compared to the control, indicating that receptor-mediated second-messenger response is increased after prenatal exposure to heroin (Abu-Roumi et al., 1996). Consistently, further studies on the signalling pathway showed a global elevation of basal membrane-bound PKC activity (Steingart et al., 1998, 2000b).

The results so far demonstrated an unexpected phenomenon of both pre- and post-synaptic hyperactivation of the components of the septohippocampal cholinergic innervation in animals exposed to heroin prenatally. However, evaluation of PKC activity after incubation of hippocampal slices with carbachol showed that this hyperactivation was associated with a complete desensitization of the receptor-mediated response (Steingart et al., 1998, 2000b). Global PKC is comprised of numerous isoforms, among which the calcium-dependent PKCs were largely implicated in behavioural function. Within these isoforms PKCγ appears particularly pertinent in learning and memory keyed to hippocampal function (Angenstein and Staak, 1997; Beldhuis et al., 1992; Bliss and Collingridge, 1993; Newton, 2001; Pascale et al., 1998). As a first step in assessing the relative role of the PKC isoforms in the heroin-induced hippocampal behavioural deficits, we quantitated PKCγ in the hippocampus of control and heroin-exposed mice offspring. Western blot analysis (Colombo and Gallagher, 2002; Colombo et al., 1997) does not differentiate any specific response of the enzyme to cholinergic stimulation as opposed to the general cellular pool. Consequently, we developed the protocol for the assessment of cholinergic receptor-stimulated PKCγ translocation by incubation with the cholinergic agonist, carbachol. Consistent with its affect on global PKC (Steingart et al., 1998, 2000b), prenatal exposure to heroin induced a complete desensitization of PKCγ to cholinergic receptor stimulation. The results of PKCγ were compared to those of PKCa, which is not related to the heroin-induced behavioural alterations and was unaffected by prenatal heroin exposure (Shahak et al., 2003).

Our findings point to a unique phenomenon which occurs after prenatal heroin exposure; global hyperactivity both pre- and post-synaptic. Conversely, this hyperactivity is associated with a complete desensitization of the cholinergic receptor-mediated response of PKC (Shahak et al., 2003; Steingart et al., 1998, 2000b). The hyperactivity appears to represent a futile attempt (because, as shown above, the behaviour, representing the net output was not corrected) to compensate for the PKC desensitization, or alternatively that the desensitization represents a ‘ceiling’ effect where PKC cannot be increased any further above the already-elevated basal levels, thus blunting receptor-mediated responses. We suggest that what is important to the proper expression of behaviour is not the level of neurochemical activity per se, but the system flexibility which enables a response to signals and therefore, the net outcome of prenatal heroin exposure is cholinergic down-regulation. This notion which is supported by our behavioural studies implies a decrease in the net output (i.e. the behaviour) of the cholinergic innervation. Human studies also support this notion that a high basal PKC level is not necessarily advantageous by demonstrating that the basal PKC level assessed by immunoblotting in Alzheimer’s patients was higher than normal in their hippocampal neurons in CA3–CA4 (Masliah et al., 1990).

While our model focuses on a specific route by which heroin administered prenatally induces specific behavioural deficits, simultaneous action of heroin on other signalling targets is also expected. Since alteration of PKC was generalized to many disparate neuroteratogens (Chen et al., 1997; Hasan et al., 2001; Haykal-Coates et al., 1998; Hussain et al., 2000; Johnson and Prohaska, 2000; Perrone-Bizzozero et al., 1998) the alterations in cholinergic innervation are actually heterologous. PKC is a common end-point for multiple signalling pathways. Therefore, alterations of PKC are likely to compromise a wide variety of neural inputs, not just those involving cholinergic pathways. Signalling proteins downstream from the receptors are a common target for neurobehavioural teratogenesis. For this reason, it is likely that such changes are not limited to PKC (Slotkin et al., 2001; Song et al., 1998).

Consequently, we studied alterations in adenyl cyclase (AC) which regulates protein kinase A (Slotkin et al., 2001). Prenatal exposure to heroin induced long-lasting elevations of AC activity. The effect was most robust with stimulants that activate AC directly (forskolin, Mn2+), indicating increased expression of AC itself; there were also shifts in catalytic properties
suggestive of a change in the AC isoform. Superimposed on the overall induction of AC, there were deficits in the responses to stimulants working through GPs or GP-coupled receptors, indicating loss of response to stimulants acting upstream from AC. Accordingly, this pattern is virtually identical to what we found for the affects of heroin on PKC (Steingart et al., 1998, 2000b), and it appears to be a common feature of apparently unrelated neuroteratogens (Buznikov et al., 2001; Hasan et al., 2001, Ferrone-Bizzozero et al., 1998).

Confirming the results of the mouse model with a well controlled model, the chick embryo

The rodent models, while indispensable in neurobehavioural teratology research, suffer methodological shortcomings mostly resulting from confounding indirect variables such as maternal physiology or mother–offspring interaction (Barron et al., 1991; Navarro et al., 1988; Sastry, 1991; Sorbian et al., 1999). In order to provide a control for the current methodological shortcomings and support the findings of the mouse model, a chick embryo model was added in our studies of heroin neuroteratogenicity. Fertilized eggs were injected with heroin (20 mg/kg) at the beginning of incubation and the filial imprinting behaviour of the chicks was tested post-hatching (Bolhuis et al., 1998, 2000). The imprinting behaviour was chosen because, like the eight-arm maze for mice, it is related to a specific area of the brain, the intermedial part of the hyperstriatum ventrale (IMHV) (Horn, 1998). Concomitant alterations in PKCγ were assessed in the IMHV. This isoform was chosen because it was implicated in learning and learning-related behaviours (Van der Zee et al., 1997). Prehatch exposure to heroin diminished imprinting behaviour and significantly reduced membrane PKCγ in the IMHV (Yanai and Metsuyanim, 2002). Therefore, the chick model, which is free from the methodological hindrances stemming from possible maternal effects of the mammalian model, provided an additional support to the mechanistic role of PKC in heroin neuroteratogenicity.

Studies on possible relationship between the biochemical deficits and the behavioural deficits

Thus, we have shown that both the biochemistry of the septohippocampal cholinergic innervation and related behaviours were impaired by prenatal heroin exposure. Concomitant biochemical and behavioural alterations do not necessarily prove a causal relationship. In fact, the causal relationship is only a relative, rather than an absolute concept. Bearing this limitation in mind, we have used the following approaches in order to assess the relative contribution of the pre- and post-synaptic components to the disruption in the cholinergic function, enabling a greater understanding of the mechanistic role in heroin neuroteratogenicity: (a) neural grafting and (b) ‘within-individual correlations’ between the behavioural deficits and alterations in components of the cholinergic cascade.

In the neural grafting study, 35-d-old mice offspring born to heroin-consuming mothers and their respective controls were grafted with normal, septal, embryonic, cholinergic cells into their hippocampus. The grafted heroin-exposed offspring showed partial reversal of the increase in the hippocampal choline transporter sites (Steingart et al., 2000b). Reversal of the heroin-induced alteration of the muscarinic receptor number has never been attempted before, nor in this model. However, in a related neuroteratogen, phenobarbital, which like heroin, also acts on the cholinergic innervation, grafting partially reversed this alteration (Rogel-Fuchs et al., 1994). Both alterations induced by prenatal heroin in the second-messenger IP and basal PKC activity were completely reversed by neural grafting, and sensitization of PKC activity to cholinergic receptor stimulation was fully restored (Steingart et al., 2000a,b). Functionally, the deficits in an eight-arm maze that were shown in the heroin-exposed animals were also reversed by grafting (Steingart et al., 2000a,b).

The second approach, ‘within-individual correlations’ between the behavioural deficits and neurochemical alterations has rarely been attempted in neurobehavioural teratology research. One hindering factor was the inability to obtain enough tissue from a specific region of an individual brain. This limitation was overcome in our studies by various approaches, for example in the case of binding, applying a single representative concentration. Simultaneously, Scatchard plots or dose–response curves were reconfirmed when applicable. The alterations in the choline transporters number, basal PKC activity and PKC desensitization were correlated within-individuals with the deficits induced by prenatal heroin in the hippocampal behaviour eight-arm maze. There were small but statistically significant correlations between the performance in the eight-arm maze and the pre-synaptic marker choline transporter, the number of hippocampal choline transporter sites having been assessed by HC-3 binding. On the other hand, the downstream post-synaptic markers’ IP formation, and the basal activity of PKC and its activity in response to muscarinic receptor stimulation, were highly
correlated with the behavioural deficits (Steingart et al., 2000a,b).

Taken together the neural grafting and the correlation studies both implicate the septohippocampal cholinergic innervation, specifically pinpointing the desensitization of PKC activation and translocation to the cholinergic receptor stimulation, as a major component in the teratogenic action exerted by heroin on hippocampus-related behaviour. However, heroin, similar to other opiate drugs, acts primarily on the opioid innervation. Therefore, we hypothesize that the effect of prenatal heroin on the behaviours under study is via the known opioidergic innervation regulation of the septohippocampal cholinergic innervation. The findings published in the literature on the changes induced by prenatal opioid exposure and on opioid regulation of cholinergic function varied greatly due to inconsistencies in experimental design and conditions. Therefore, it is still premature to put forth a conclusive statement regarding the direction of the changes and effects: increases or decreases. Yet, the data gathered thus far has pointed to the existence an effect of prenatal exposure to heroin and the opioidergic innervation and that there is opioidergic regulation of cholinergic function. Prenatal exposure to morphine, when the treatment was terminated prior to birth, induced an overall increase in the function of the opioidergic innervation. Specifically, studies on endogenous opiates suggest that prenatal morphine exposure caused a decrease in their content in the presynaptic terminals (Tempel et al., 1995; Tiong and Olley, 1988). It was argued that this change actually reflects an increase in the release that represents presynaptic hyperactivity. This notion is supported by the increase in proenkephalin mRNA expression (Tempel et al., 1995). Studies on opiate receptors suggest that there are post-synaptic adaptive changes in the opiate receptors: hypoactivity, possibly to offset the presynaptic alteration (Hammer et al., 1991; Kirby and Aronstam, 1983; Tempel, 1991; Tempel et al., 1988; Tsang and Ng, 1980). Our findings on the partial agonist, buprenorphine, supported these results by demonstrating hypoactivation of \( \mu \)-receptors after prenatal exposure to the drug (Belcheva et al., 1994, 1998). All alterations, pre- and post-synaptic, induced by prenatal exposure to opioids are only short term as they never lasted beyond several days after the drug exposure was terminated (Belcheva et al., 1994; Kirby and Aronstam, 1983; Tempel, 1991; Tempel et al., 1988). Since prenatal exposure to opioids has different effects on the pre- and post-synaptic components of the opioid innervation, functional studies, which ascertain the net output, will determine whether overall hyper- or hypoactivation has occurred. Although some findings to the contrary are also available (O’Callaghan and Holtzman, 1976), the weight of the evidence leans towards enhanced analgesic response. Accordingly, several groups have shown an increased analgesic response to morphine in rat offspring exposed to morphine prenatally (Castellano and Ammassari-Teule, 1984; Gagin et al., 1996).

We hypothesize that in the present model heroin exerts its neurobehavioural teratogenic effect as follows: prenatal exposure to heroin induces an overall increase in the function of the opioid innervation as described above which, as will be argued, exerts a regulatory action to decrease the function of the septohippocampal cholinergic innervation, resulting in the deficits in hippocampus-related behaviours. Opioids are thought to have a modulatory role on various transmitters. Overwhelming evidence is available on opioid regulation of the cholinergic innervations, and although results vary depending on the experimental conditions and parameters (Kearns et al., 2001), certain patterns can emerge. It appears that activating the \( \mu \)-receptors inhibits cholinergic activity and function assessed by cholinergic-related learning tasks. Thus, morphine, a \( \mu \)-preferring agonist, inhibited ACh release in several CNS regions (Beani et al., 1982) including the hippocampus (Lapchak et al., 1989). In the striatum, the inhibition occurred via \( \mu \)- and \( \delta \)-receptors but not via \( \kappa \)-receptors (Arenas et al., 1990; Sandor et al., 1992). Functional (behavioural) studies were consistent in that antagonists, and not agonists, of \( \mu \)- and \( \kappa \)-opioid receptors improve performance in learning tasks (Collier and Routtenberg, 1984; Gallagher et al., 1983; Messing et al., 1979).

Conclusions

Similar to other opioids, heroin has a generalized effect on the brain not limited to the opioid innervation, and therefore an effective approach in the study of the mechanism of heroin neurobehavioural teratogenicity is to establish a model of the drug effect on the ’region and innervation specific behaviours’, which is the subject of the present paper. Unlike most similar models which employed morphine, assuming that it represents the heroin action, we argue for the employment of heroin due to the recent evidence of its unique action. Prenatal exposure of heroin in mice induces deficits in the eight-arm and Morris maze behaviours which are related to septohippocampal cholinergic innervation, as well as in the correlated cholinergic cascade. In contrast to what is known on manipulating the adult brain where presynaptic

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alterations usually evoke compensating post-synaptic responses and vice versa, prenatal exposure to heroin resulted in a unique global hyperactivity both pre- and post-synaptic in the septohippocampal cholinergic innervation. These alterations were accompanied by desensitization of PKC activity in response to cholinergic agonist which represents either the cause or the outcome of the global hyperactivity. Our findings, taken together with what is known from the literature, bearing in mind the inconsistencies therein, yield the following hypothesis. Prenatal exposure to opioid agonists appears to induce opioidergic hyperactivity. This is because, while there was a general presynaptic hyperactivity with seemingly post-synaptic hypoactivity of the receptors, the net functional outcome as indicated by the altered analgesic response was a functional enhancement. This alteration in the opioidergic innervation attenuates cholinergic activity and function resulting in the abnormalities in the septo-hippocampal cholinergic innervation and in its behavioural output as shown in our model.

Our model provides a comprehensive account of heroin teratogenicity beyond its action on the opioid receptors via the alteration in the hippocampal cholinergic innervation to the behavioural deficits. Furthermore, the findings of the model enable the reversal of the deficits with neural grafting. This information thus obtained provides the groundwork for further studies on the reversal of the deficits caused by narcotic addiction by more clinically feasible means, as well as providing for the prevention of the heroin insult in those cases when the intake during pregnancy cannot be avoided.

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