Developing hypothalamic dopaminergic neurones as potential targets for environmental estrogens

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Abstract

Environmental chemicals which mimic the actions of estrogen have the potential to affect any estrogen responsive tissue. The aim of the present study was to investigate their potential to mimic the effects of 17 β -estradiol (E₂) on developing primary rat hypothalamic dopaminergic (DA) neurones maintained in a chemically defined medium. We now show that both E₂ and octylphenol (OP), but not the non-aromatizable androgen, dihydrotestosterone, enhanced the uptake of [³H]DA by the cultured cells, whereas they had no effect on the uptake of [¹⁴C]GABA. Although the sensitivity of responses may change with the age of the developing cultures, the dose response

curves for E₂ and OP were typically 'bell-shaped', with a rise in response followed by a decline to control levels with increasing concentrations. Effects were seen as low as 10⁻¹⁴ M for E₂ and 10⁻¹¹ M for OP. Responses to E₂ (10⁻¹² M) and OP (10⁻⁹ M) were reversed in the presence of the antiestrogen, ZM 182780 (10⁻⁵ M). This study thus provides direct evidence, using a mechanistic rather than toxicological end-point, in support of the hypothesis that inappropriate exposure to environmental estrogens at critically sensitive stages of development, could potentially perturb the organisational activities of estrogen on selected neuronal populations in the CNS.

Introduction

There is growing concern that exposure to environmental chemicals which mimic the actions of estrogen have the potential to disrupt endocrine function and thus pose a threat to health (Sharpe 1993, Birmbaum 1994, Feldman 1997). While this link is not proven, many studies now document the ability of these chemicals to interfere with the actions of steroid hormones in wildlife (Colborn & Clement 1992, Colborn *et al.* 1993, Guillelte *et al.* 1996) and in experimental animals (Malby *et al.* 1992, White *et al.* 1994, Steinmetz *et al.* 1997, 1998, vom Saal *et al.* 1998). Increasingly attention is focused on early life stages when steroid hormones play a crucial role in the development of many tissues and organs which undergo distinct periods of enhanced developmental sensitivity. This has been demonstrated in the periphery, for example, where testicular development and sperm production are impaired in the adult offspring of rats fed with xenoestrogens (octylphenol, OP, or bisphenol A, BPA) through gestation and lactation (Sharpe *et al.* 1995, vom Saal *et al.* 1998). Less is known, however, about the potential effects of such compounds in the central nervous system which is exquisitely sensitive to estrogen exposure at critical periods in early life. The hypothalamus is potentially a prime target where it has been shown that sex differences in the control of neuroendocrine functions, such as the regulation of reproductive behaviours and secretion of gonadotrophins, is programmed perinatally in the rat by testosterone acting via an estrogen receptor (ER)-mediated event after its conversion to

17 β -estradiol (E₂) by aromatase enzymes located centrally (MacLusky & Naftolin 1981).

In support of a hypothalamic site of action of potential endocrine disrupting chemicals, *in vivo* studies have demonstrated disturbances in reproductive neuroendocrine function and behaviour in the offspring of pregnant rats fed with low doses of 2,3,7,8-tetrachlorodibenzo-p-dioxin (Malby *et al.* 1992, MacLusky *et al.* 1998 and references therein). There is controversy, however, as to whether the drug treatment could also compromise the perinatal rise in testicular androgen secretion and, hence, affect the central masculinization/defeminization processes. Thus, it is unclear whether interference with neuroendocrine development resulted from direct actions within the brain or was secondary to a peripheral effect. Therefore, in this study, we have adopted a cell culture approach with the aim of investigating directly whether xenoestrogens could influence developing hypothalamic neurones. We have focused on OP, the most estrogenic of the environmentally persistent alkylphenolic compounds widely used in industry (White *et al.* 1994), and how it affects hypothalamic dopaminergic (DA) neurones. These cells have established roles in neuroendocrine regulation (Mackenzie *et al.* 1988, Zorilla *et al.* 1990, Abrogast *et al.* 1990, Wilson *et al.* 1991) and many express estrogen receptors (Sar 1984, Herbison & Theodosis 1992). Furthermore, they are sensitive to perinatal manipulations of the sex steroid hormone environment both *in vivo* (Demarest *et al.* 1981, Simerley *et al.* 1989) and as they develop in culture (Murray & Gillies 1993) via ER-dependent mechanisms (Simerley *et al.* 1997).

Materials and Methods

Culture preparation and treatments

Primary hypothalamic cell cultures were prepared from embryonic day 18 CFY rat hypothalami as described in detail elsewhere (Clarke & Gillies 1988, Murray & Gillies 1993) and plated at equal density (0.5×10^6 cells/well/200 μ l) in 15 mm diameter tissue culture wells pre-coated with poly-L-lysine (10 μ g/ml). Cultures were maintained in a chemically defined, serum- and phenol red-free medium consisting of a 1:1 mixture of Dulbecco's Modified Eagles Medium and Ham's F12 nutrient medium (Life Technologies) supplemented with triiodothyronine (1 nM), putrescine (0.1 mM), selenium (30 nM), insulin (5 μ g/ml), human apo-transferrin (100 μ g/ml), penicillin (100 IU/ml), streptomycin (100 μ g/ml) and fungizone (0.5 μ g/ml) (Sigma, Poole, Dorset, UK). No further supplements were added to the medium (controls), or a range of concentrations of E₂ or OP (Batch no. 113311-035, Sigma, Poole, UK) were added from the time of plating. In selected experiments the anti-estrogen, ZM 182780 (gift from Zeneca Pharmaceuticals), was also present from the time of plating. Medium was changed every 3 days and vehicle controls

(ethanol) were negative. When used, cytosine arabinoside (10^{-5} M) was added to the culture medium 24 h after plating and removed 48 h later (Davidson & Gillies 1993).

Uptake studies

The uptake of [³H]DA was determined at intervals over 2-3 weeks *in vitro* as an indicator of maturing responses which parallel *in vitro* developmental changes in DA neurone morphology and endogenous DA content and release (Murray & Gillies 1993). A limited number of similar studies were performed for [¹⁴C]GABA uptake. Cultures were first rinsed three times with 200 μ l of pre-warmed Earle's Balanced Salt Solution (EBSS) and then pre-incubated for 15 min with 200 μ l of EBSS supplemented with penicillin/streptomycin, as above, bovine serum albumin (0.1%), ascorbic acid (30 μ g/ml), HEPES (10 mM), and aprotinin (80 kIU/ml). The cells were then exposed for 1 h at 37°C to 200 μ l of [³H]DA (specific activity approx. 50 Ci/mmol, Dupont Ltd, NEN Life Science Products, Hounslow, UK) at a concentration of 3.3×10^{-7} M (approximately 2 μ Ci per well depending on specific activity) diluted in EBSS plus additives for in the presence of the monoamine oxidase inhibitor pargyline (10 μ M), or to

Figure 1 Effects of increasing concentrations of 17 β -estradiol and octylphenol on the uptake of [³H]-dopamine into foetal rat hypothalamic cells in primary culture. Results represent the mean $\pm$ S.E.M., $n=4$, * $P<0.05$ compared with control (□) using Student-Newman-Keuls method. Abscissa, logM of compound.

Figure 2 Absence of effect of increasing concentrations of 17β-estradiol on the uptake of [¹⁴C]GABA into foetal rat hypothalamic cells in primary culture on day 9 *in vitro* (DIV). Similar results were obtained on DIV 11 and 14. Results represent the mean±S.E.M., *n*=4. Abscissa, logM of compound.

[¹⁴C]GABA (NEN, DuPont) at a concentration of 5.6×10^{-8} M (approximately 0.15 μCi per well), for 10 min. in the presence of the GABA-transaminase inhibitor, amino-oxyacetic acid (10^{-5} M). Uptake was terminated by removal of the radioligand, followed by 3 successive washes with 200 μl of ice cold EBSS accompanied by gentle shaking. The EBSS was then replaced with 200 μl of 0.5 M perchloric acid (PCA) and the cells were removed from the substratum after scraping with a 1000 μl pipette tip. The PCA was transferred to a scintillation vial and each well was rinsed with an additional 100 μl of PCA to ensure transfer of all the radioactivity to the scintillation vials. 5ml of scintillation fluid (HiSafe 3, Fisher Scientific, Loughborough, UK) was added to each vial and the accumulated radiolabelled neurotransmitter was estimated by liquid scintillation spectrophotometry. Non-specific uptake was approximately 20% of total for DA, as determined in the presence of 10^{-4} M benztropine (Murray & Gillies 1993), and <10% for GABA, as determined in the presence of 10^{-5} M SKF 89976A (Gillies & Davidson 1992). Non-specific uptake of [³H]-DA into norepinephrine (NE) or 5-hydroxytryptamine (5HT) producing neurones is unlikely as the NE and 5HT present in the hypothalamus *in vivo* arises from cell bodies outside the hypothalamus (Daikoku *et al.* 1986) and, furthermore, desmethylinipramine (blocker of uptake into noradrenergic terminals) and fluoxetine (blocker of uptake into serotonergic terminals) have no effect on DA uptake (Puymirat *et al.* 1983, Fuller *et al.*, 1994). Preliminary experiments established that protein content (Bradford 1976) was not

significantly affected by treatments e.g. in the absence or presence of E₂ (10^{-12} M) 13.26 ± 2.46 μg and 14.29 ± 3.62 μg of protein per well were found, respectively, on day 12 *in vitro* and 17.34 ± 1.29 μg and 17.76 ± 0.93 μg of protein per well were found, respectively, on day 16 *in vitro* (means ± S.E.M., *n*=4). Furthermore, the DA neurone population represents less than 1% of cultured hypothalamic neurones (Puymirat *et al.* 1983). Therefore, changes in protein content are unlikely to indicate specific changes in the DA neurone population but are more likely to be due to changes in the glial cell population which proliferates, albeit at a much reduced rate compared to

Figure 3 Antagonism of the effects of: A, 17β-estradiol (E₂, 10^{-12} M) and B, octylphenol (OP, 10^{-9} M) on [³H]-dopamine (DA) uptake into foetal rat hypothalamic cells in primary culture by the antioestrogen ZM182780 (ZM, 10^{-5} M) on day 9 *in vitro*. Results represent the mean±S.E.M., *n*=4. **P*<0.01, ***P*<0.001, Students *t*-test.

cells maintained in serum-supplemented medium (Clarke & Gillies 1988). Therefore, uptake is expressed as pmoles per well.

Statistical analysis

Statistically significant differences were determined using analysis of variance (ANOVA) and, where a significance level of $P < 0.05$ was achieved, this was followed by the Student-Newman-Keuls multiple range test or the Student's *t*-test. Results are expressed as means $\pm$ S.E.M. of four replicates; data are representative of at least three separate experiments.

Results

Over the first few days in culture (DIV 4, Fig. 1) no effects were seen in response to E_2 or OP. This confirms our earlier findings with E_2 and may be due to an adaptation to culture conditions and/or changing developmental sensitivity (Murray & Gillies 1993). However, by day 9 *in vitro* both E_2 and OP caused a significant increase in [3H]DA uptake and this was maintained through to the later stages in culture (Fig. 1). The dose-response curve was typically 'bell-shaped', with a gradual rise in response followed by a decline to control levels at the highest concentration tested. In some instances, however, responses appeared to be near maximal even at the lowest concentration tested (e.g. DIV 13 for E_2). Similar effects were not seen on the uptake of [^{14}C]GABA, which was unchanged in the presence of E_2 (Fig. 2) or OP (10^{-12} - 10^{-6} M; data not shown). In addition, the non-aromatizable androgen, dihydrotestosterone (DHT, 10^{-12} - 10^{-6} M), did not enhance the uptake of [3H]DA (data not shown). Figure 3 shows that the antiestrogen, ZM 182780 (10^{-5} M), completely reversed the effects of E_2 (10^{-12} M) and OP (10^{-9} M).

Taken together, these data provide evidence for a selectivity of effects of estrogenic compounds on DA neurones within these cultures which appears to involve an ER-mediated pathway.

In order to dissociate possible effects of estrogenic compounds on uptake into neuronal and glial cells, total and non-specific uptake of [3H]DA, as determined in the absence and presence of a maximally effective concentration of a DA uptake inhibitor (10^{-4} M benztropine; Murray & Gillies 1993), was investigated in the absence and presence of cytosine arabinoside (10^{-5} M) which arrests glial cell proliferation (Davidson & Gillies 1993). The data in Table 1 indicate that the glial cell population could account for 30-40% of the non-specific uptake. However, this did not change as a function of time in culture between days 8 and 17 or in response to treatments with E_2 or OP, suggesting that the effects of the estrogenic compounds were not due to effects on glial cells.

Discussion

This study provides direct evidence for a selectivity of effects of a natural and a xenobiotic estrogen on developing

Table 1 Contribution of glial cells to [3H]DA uptake. Uptake was determined on day 8, 11 14 and 17 *in vitro* (DIV) without or with cytosine arabinoside treatment (no C.A. and plus C.A., respectively) and the glial component is the difference between the two. Determinations were in the absence (0) or presence of 17β -estradiol (E_2 , 10^{-12} M) or octylphenol (OP, 10^{-9} M). Results represent the mean $\pm$ S.E.M.

	Non-specific uptake of [3H]DA (% of total)		
	- C.A.	+ C. A.	Glial component
DIV8			
0	22.40 $\pm$ 3.85	16.32 $\pm$ 2.96	6.09 $\pm$ 3.84
E_2	19.78 $\pm$ 3.04	11.81 $\pm$ 2.32	8.12 $\pm$ 3.03
OP	20.69 $\pm$ 3.40	16.55 $\pm$ 1.79	4.14 $\pm$ 3.40
DIV11			
0	19.42 $\pm$ 2.46	14.27 $\pm$ 2.04	5.15 $\pm$ 2.45
E_2	22.90 $\pm$ 1.59	15.68 $\pm$ 1.02	7.22 $\pm$ 1.59
OP	20.81 $\pm$ 3.32	13.76 $\pm$ 2.72	7.05 $\pm$ 3.31
DIV14			
0	22.68 $\pm$ 3.90	14.64 $\pm$ 1.64	8.03 $\pm$ 3.89
E_2	20.43 $\pm$ 3.67	12.32 $\pm$ 2.57	8.11 $\pm$ 3.67
OP	19.57 $\pm$ 4.02	9.55 $\pm$ 1.60	10.02 $\pm$ 4.01
DIV17			
0	22.25 $\pm$ 2.97	14.42 $\pm$ 2.22	7.83 $\pm$ 2.97
E_2	22.68 $\pm$ 3.81	16.19 $\pm$ 2.74	6.49 $\pm$ 3.81
OP	21.60 $\pm$ 4.52	11.04 $\pm$ 3.39	10.56 $\pm$ 4.52

hypothalamic DA neurones. To the best of our knowledge, this is the first report of a functional, neurochemical response to an environmental estrogen. Preliminary results show, also, that the xenoestrogen bisphenol A (BPA) as well as the phytoestrogen, genistein, at 10^{-9} M also increase the uptake of [3H]DA to $154.4 \pm 6.5\%$ and $165.34 \pm 8.1\%$, respectively, relative to controls (means $\pm$ S.E.M., $n=4$, $P < 0.01$; unpublished data). The data thus support the hypothesis that inappropriate exposure to environmental estrogens at critical stages of development could potentially perturb the organisational activities of endogenous estrogen on selected neuronal populations in the CNS.

Antagonism of the effects of E_2 and OP by ZM 182780 supports other data indicating that the environmental estrogens may be acting via the intracellular ER (White *et al.* 1994, Kuiper *et al.* 1997, 1998, Pennie *et al.* 1998). While the sensitivity of response may change with the age of the developing cultures, we have observed effects at 10^{-14} M for E_2 and 10^{-11} M for OP. This is in keeping with other findings that *in vitro* the xenoestrogens are several orders of magnitude less potent than E_2 (White *et al.* 1994, Steinmetz *et al.* 1997, Kuiper *et al.* 1997, 1998), although the sensitivity of the cultured cells appears extremely high. Significant effects of low dose E_2 and BPA have also been reported on prolactin release from cultured anterior pituitary cells obtained from adult rats of the estrogen-sensitive Fisher rat strain (Steinmetz *et al.* 1997). Whilst prolactin release elicited by the lowest dose of BPA tested (10^{-8} M) was not much greater than control, the response to the

lowest dose of E_2 (10^{-12} M) was almost half maximal. It is possible, therefore, that other tissues might also have a great sensitivity to ER ligands. However, interpretation of the implications of these studies for the whole animal requires, amongst other things, a better understanding of the mechanisms which may regulate the bioavailability, at a tissue specific level, of estrogenic compounds, whether they are of an endogenous or environmental origin. For example, in placental mammals the foetus is exposed to relatively high levels of maternal and placental estrogens and it has been estimated in mice that the circulating levels of free E_2 are around 10^{-12} M (vom Saal *et al.* 1997). However, it is thought that the developing brain is protected from circulating E_2 but not testosterone. This is thought to be due largely to a binding protein identified in rats and mice as alpha-fetoprotein (AFP) which prevents E_2 crossing the blood-brain barrier (MacLusky & Naftolin 1981). An AFP-like substance has also been identified in the brain itself where there is a striking regional specificity in its localisation within certain developing nerve cells, but not in those cells capable of accumulating tritium-labelled E_2 or testosterone (Toran-Allerand 1980). The mechanism of protection is less clear in humans. The actual exposure to the environmental estrogens is still in debate (Feldman 1997), but it seems likely that they escape the protective mechanisms developed for E_2 .

Much of our current understanding of the sensitivity and relative activities of ER ligands derives from cell-based systems used in transcription assays and ligand binding studies. Although methods vary, such experiments (see for example Kuiper *et al.* 1997, 1998) predict a dissociation constant for E_2 -ER around 10^{-10} M, with the half maximal concentration for E_2 activation of transcription of a reporter gene occurring around 5×10^{-12} M - 5×10^{-11} M. It is now understood that, in addition to ligand concentration and the possible protection mechanisms discussed above, many intracellular factors are critical in determining the ER-mediated responsiveness in specific cell types. These include the relative expression of the α and β forms of the receptor (Paech *et al.* 1997), the expression of co-repressors and enhancers of transcriptional activation (Parker 1993) and the nature of the estrogen response element (Paech *et al.* 1997; Pennie *et al.* 1998). However, the precise nature of these factors in a given cell type is unknown and, therefore, they cannot be reproduced in cell-line based systems. In contrast, our results have been obtained using primary CNS cells expressing the native complement of response elements and accessory proteins which would affect ER sensitivity. They thus raise the possibility that environmental estrogens could influence developing CNS neurones at concentrations considerably below their accepted toxic levels at times when these cells are exquisitely sensitive to estrogenic compounds. These observations thus support the view that potential environmental endocrine disrupters should be examined at physiologically relevant concentrations, especially during development, with experimental designs

which accommodate responses exhibiting a bell-shaped dose-response curve (vom Saal *et al.* 1997, 1998).

In summary, our data suggest that environmental estrogens have the potential to act directly upon specific hypothalamic neurotransmitter systems involved in neuroendocrine control at a critical period of development. Such actions could thus contribute to the effects which environmental chemicals have on reproductive tract development (Sharpe *et al.* 1995, vom Saal *et al.* 1997, 1998) and/or permanently alter neuroendocrine function (e.g. early onset of puberty or the menopause) and reproductive behaviours in later life (Clarkson *et al.* 1995, Malby *et al.* 1992, MacLusky *et al.* 1998). Further work will be needed to determine the physiological significance of these findings, along with a better understanding of actual exposure levels and the way in which age and sex may influence biological potency of endogenous and environmental estrogens in specific tissues.

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