Behaviour modulation and expression of N-methyl D-aspartate receptor genes in adult female mice after a chronic administration of Benzo(a)pyrene

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Introduction

Environmental neurotoxins such as benzo(a)pyrene produce a variety of behavioural deficits specific to the nervous system, including decreased motor activity, neuromuscular, physiological and autonomic abnormalities[1,2]. While the carcinogenicity of Polycyclic Aromatic Hydrocarbon including B(a)P is well established, the neurotoxic effect of B(a)P has not received much attention. Recently, some authors predicted the neurotoxic potential of PAH particles deposited in the Central Nervous System, and their subsequent adverse effects on development [3]. Currently, the relationship between B(a)P and the neurotransmitter receptors has received little attention. Among these receptors, it is accepted that the glutamate receptors (the major excitatory neurotransmitter in mammalian central nervous system) in particular the N-methyl D-aspartate (NMDA) receptors play a fundamental part in the cerebral development and synaptic plasticity[4]. An excessive activation of these receptors can lead to excitotoxic damage, which has been implicated in a number of neurodegenerative disorders[5].

In situ hybridization experiments in rats revealed that the two subunits of the NMDA receptor (NR1 and NR2) have been shown to have different expression profiles. The expression of NR1 has been shown to be widespread, whereas the 4 possible NR2 (NR2a, NR2b, NR2c, NR2d) shown various temporal expression profiles[6]. Mice devoid of normal cellular prion protein, overexpressing the NR2b subunit are be able to memorize actions more effectively. The Morris water maze test reveals that these animals overexpressing the NR2b subunit also present better cognition results than controls[7]. NMDA receptors seem to have a direct link with the synaptic plasticity of the hippocampus. Indeed, the high quantity of NR2a and NR2b mRNA reveals an overexpression of the glutamate receptors in hippocampus of mice. Thus, the glutamate transfer would be facilitated and would allow a better plasticity[7]. Glutamate is an excitatory amino acid, in case of deregulation (eg cerebral ischaemia or epilepsy), it has been shown to play a role at the cerebral level in apoptosis and neuronal loss. We hypothesis that the cerebral toxicity of B(a)P, subsequent modifications in the organisation and the working of glutamatergic system are linked. This hypothesis may be partially confirmed by the work of Wormley et al. who showed that the NR2a and NR2b subunit mRNA decrease when the rats are exposed with B(a)P (100 µg/m3) during an antenatal period[8]. The level NR1 subunit mRNA was remained unchanged between the control and the groups exposed to B(a)P.

To our knowledge, there is no information concerning B(a)P exposure and the effect on NMDA genes expression in adult mammals. We have focused our work on the modulation of neurotoxic behaviour and expression of N-methyl D-aspartate receptors genes in adult female mice after chronic B(a)P administration. Initially, we studied the effect of an increasing amount of B(a)P on the expression of NMDA receptors (NR1 subunit), and subsequently, we have evaluated the relationship between the expression of NMDA receptors genes, the behaviour and B(a)P exposure.

Materials and Methods

Animals

Sixty females Balb/c mice, weighing approximately 18-20g (6 weeks of age), were obtained from Charles Rivers Laboratories, France. Animals were housed in plastic cages and acclimatized to the animal care facility for 1 week. The animal house was maintained under a timed 12h/12h light/dark cycle (light on at 7 pm) with a temperature range of 22 ± 2 °C and a relative humidity 40 ± 5 %. Mice had access to food and water *ad libitum*. Six groups were used and each mouse was specifically labelled.

Benzo(a)pyrene administration

The dosing solution was freshly prepared every week by dissolving a desired quantity of B(a)P (> 97% purity, Sigma Aldrich Co., St Quentin Fallavier, France) in vegetable oil. Mice were randomly assigned to one of the following six treatment groups (n=10/group): 0, 0.02, 0.2, 2, 20, 200 mg/kg of B(a)P. Each animal received a daily intra-peritoneal injection of B[a]P solubilized in vegetable oil (10 ml/kg body weight) on 10 consecutive days. Control animals received vegetable oil only.

Neurobehavioural analysis

Behavioural effects of B(a)P were evaluated using the anxiety (Elevated plus-maze) and the memory (Y maze, Morris water maze) tests on day 10 and the animals subsequently sacrificed. Hippocampal regions were immediatly excised and stored at -80°C untill analysis.

RNA isolation, reverse transcription and Quantitative PCR

Total RNA was purified on 10mg of homogenized tissues using a High Pure RNA isolation kit (Roche, Germany). First-strand synthesis of total cDNA was carried out at 50 °C for 60 minutes using 200 U SuperScript III RT (Invitrogen, Paisley, UK) and 2.5 μM dT_{16} primer in a 40 μl reaction containing 250 mM Tris-HCl (pH 8.3 at room temperature), 375 mM KCl, 15 mM $MgCl_2$, 10 mM dithiothreitol and 500 μM deoxynucleoside triphosphates (dNTPs).

Amplification of cDNA by PCR was performed using 20 mM Tris HCl (pH8.4), 50 mM KCl, 200 mM deoxynucleoside triphosphates (dNTPs), and 2.5 U Platinum *Taq*DNA polymerase (Invitrogen), (35 cycles: 96°C, 20 sec; annealing, 20 sec; 72°C, 20 secs), followed by 10 min, 72°C on an Opticon 2 (MJ Research). Annealing temperatures, primer sequences and $MgCl_2$ concentrations are shown in the table below:

Gene		Sequence	Product Length	Annealing Temperature	[Mg^{2+}]	[Primers]
β-actin	Fwd	CAATAGTGATGACCTGGCCGT	138	50°C	3 mM	0.5 uM
	Rev	AGAGGGAAATTGTGCGTGAC				
NMDA	Fwd	GAGGCTTGGGCTTGTGGGTGA	222	64°C	2 mM	0.5 uM
NR1	Rev	ATGGCGTTGGGCTTGTGGGTGA				

Specific pCR4-TOPO clones were used to generate absolute calibration curves. Plasmids were sequenced using 100 nM M13 primers and the BigDye 3.1 terminator cycle sequencing reagent (Applied Biosystems, Nieuwerkerk, NL) to ensure the correct sequence insert. Plasmid DNA was linearised using *Not I* and quantified using PicoGreen. Q-RT PCR results are expressed as the number of copies of the receptor per 10^2 copies of β-actin.

Results and Discussion

One way analysis of variance of all groups revealed a significant treatment effect on the weight at the end (p=0.027) of the experiment compared to their initial weight. In fact, the mice treated at high concentration of B(a)P (200mg/kg) showed a significant weight decrease (p=0.02). However, there was no significant difference in the brain/body weight ratio between the control and experimental groups, which were determined by analysis of the brain weight at sacrifice on day 10.

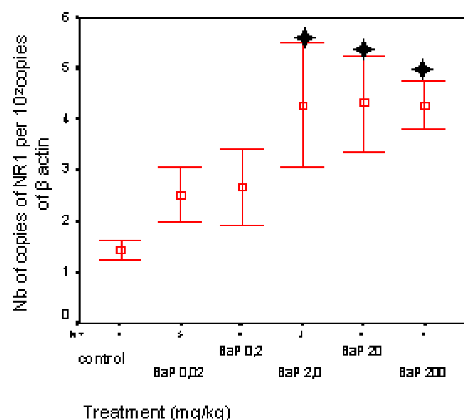

Figure 1. mRNA analysis of the NR1 subunit of the NMDA receptor after a chronic administration of B(a)P ö P < 0.05 difference statistically significant versus control group (Dunnett test)

In the present study, we showed that adult mice chronically expose to B(a)P increased NR1 mRNA levels in the hippocampus (Figure 1). This result shows a dose-reponse relationship between the level of B(a)P exposure and the expression of NR1 gene in this brain region (ANOVA, p=0.042). The Dunnett *post hoc* multiple comparison procedure showed a significant increase more than 3 times of NR1 expression at 2, 20 and 200 mg/kg compare to the control (p<0.05). This result led us to suppose that B[a]P could alter the regulation of glutamatergic neurotransmission. The mechanisms by which chronic exposure to different levels of B(a)P (0.02 to 200 mg/kg) increases the overall expression of NR1 mRNA is not currently know.

However, other studies carried out on other pollutants like Pb^{2+}, reveal that these molecules also cause an overexpessing of sub-unit NR1 in the pyramidal cells of the hippocampus of young rats[9]. The measure of NR1 mRNA could be a relevant biomarker to neurotoxicity range from the effect of B(a)P.

Moreover, this study has showed some neurobehavioral effects of B(a)P on anxiety and memory in adult female mice. Anxiety was measured using the elevated plus-maze.

B[a]P induced an obvious and significant anxiolytic effect at 200 mg/kg (p<0.05 versus control), as shown by the time spent in the non protected zone (open arms, Figure 2). This reduction of anxiety at high concentration confirms

anterior results where rats were contaminated by B(a)P at 20 mg/kg during their development period[9]. However, we can see that B(a)P does not change the overall activity in mice. This non significant difference on overall activity between the treated and control groups was also demonstrated in the Y maze by the Kruskall-Wallis test.
Immediate working memory performance was assessed by recording spontaneous alternation behaviour in a Y-maze. The statistical analysis conducted with the Kruskall-Wallis test confirms a significant decrease of short-term memory (p=0.053). Mann-Withney analysis reveals that the mice treated at low levels 0.002 and 0.2 mg/kg learn less than the control group (p=0.11 and p= 0.014 respectively, Figure 3). These results are confirmed by the Morris water maze test, which shows after 5 training trials a significative deficit of learning in treated groups versus the control group (p=0.032).

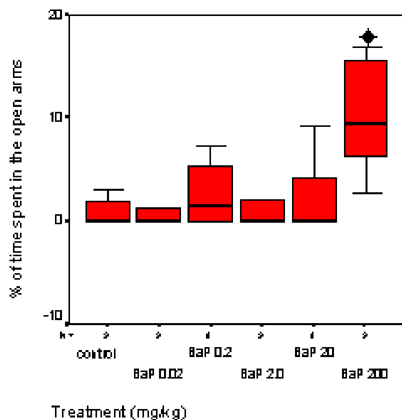

Figure 2. Evaluation of the anxiolitic behaviour of mice after a chronic administration of B(a)P using Elevated plus-maze.
ö P< 0.05 difference statistically significant versus control group (Mann-Withney test)

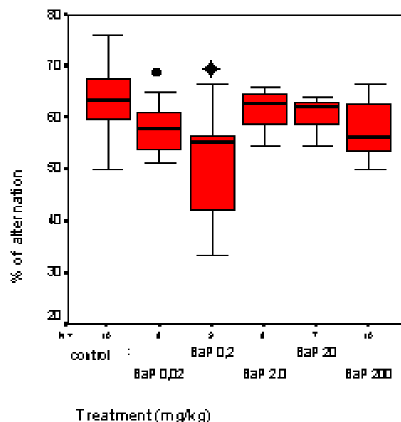

Figure 3. Evaluation of the short term memory performance in mice chronically exposed to B(a)P using the Y-maze test
ö P< 0.05 difference statistically significant versus control group (Mann-Withney test)
• p = 0.11

The modulation of behaviour observed between the low doses (deficit on short term memory and learning) and the high doses of B(a)P (weight decrease and anxiolytic effect) could be explained in part by the induction of cytochrome P450. In fact their induction linked to the high dose of B(a)P in brain tissues such as the hippocampus could modify the BaP metabolites quantities and profile in the brain. This hypothesis may be partially confirmed by the work of Saunders et al.[1] who showed a dose-response relationship between B(a)P doses and metabolite levels in rat brain. The formation of reactive metabolites (including the 7,8-diol-9,10-epoxide) could lead to other behavioural effect such as the anxiolitic effect observed. Analysis of the metabolites is currently underway in our laboratory to confirm this hypothesis.

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