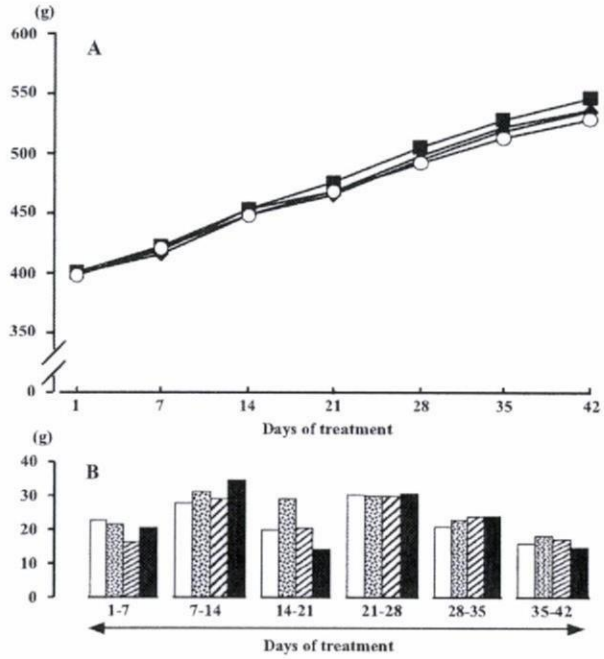

Fig. 3. Changes in body weight (A) and its gain (B) in male rats treated orally with 4-methylbenzoic acid for 42-days at dose level of 0 (○ and open column), 100 (■ and dashed column), 300 (▲ and hatched column) or 1,000 mg/kg/day (♦ and closed column) in the reproduction/developmental toxicity screening test. Each value represents the average for 13 animals.

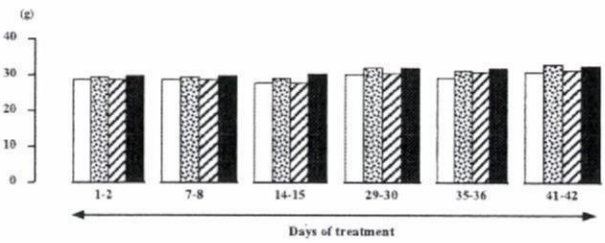

Fig. 4. Changes in food consumption of male rats treated orally with 4-methylbenzoic acid for 42-days at dose level of 0 (open column), 100 (dashed column), 300 (hatched column) or 1,000 mg/kg/day (closed column) in the reproduction/developmental toxicity screening test. Each column represents the average for 13 males.

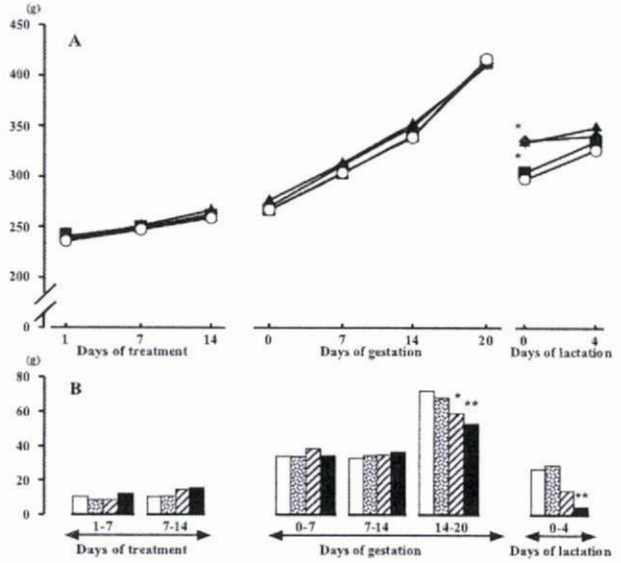

Fig. 5. Changes in body weight (A) and its gain (B) in female rats treated orally with 4-methylbenzoic acid at a dose level of 0 (○ and open column), 100 (■ and dashed column), 300 (▲ and hatched column) or 1,000 mg/kg/day (♦ and closed column) in the reproduction/developmental toxicity screening test. Administration of the compound was started 2 weeks prior to mating, and was continued through mating period and gestation period until 3 days after delivery. Each value during the pre-mating treatment period represents the average for 13 animals. That during the gestation and lactation periods represents the average for 9-13 dams. * and ** indicate significant difference from control at $p < 0.05$ and 0.01, respectively.

control. Males in the groups given 300 mg/kg or less did not show such abnormalities at any part of their epididymis (Table 6). In the testis, no abnormal findings related to the doses of the compound were observed.

Reproductive performances in the reproduction/developmental study

Except one female in the control and one female in the 100 mg/kg treated groups, all the females revolved on a regular 4-day estrous cycle until mating (data not shown). Mating performance and dam data are shown in Table 7. None of indices for mating performance were different between the compound treated group and the control group. In addition, the number of corpora lutea, which represented the number of oocytes shed for impregnation, was not different between these groups. Thus, the compound did not affect the estrous cycle, ovulation or mating, at any dose level.

Although all of the females that had copulated in the 100 mg/kg and less treated groups became pregnant, one such female and four such females in the 300 mg/kg and 1,000 mg/kg treated groups, respectively, did not become pregnant (Table 7). The Fisher's direct probability test indicated that there was a significant difference in the fertility between the control and the 1,000 mg/kg treated groups, which indicated that the compound disrupted fertility at 1,000 mg/kg.

As shown in Table 7, pregnant females delivered live fetuses without differences in gestation length between the control and the compound treated groups. However, the implantation index and the number of pups born were significantly decreased in the 300 mg/kg or more treated groups. Furthermore, the numbers of pups alive on Days 0 and 4 of lactation were significantly smaller in the 1,000 mg/kg treated group than those in the control. The other dam data, such as the birth index, the live birth index and the viability index on Day 4 of lactation, were not affected by the treatment. In addition, pup body weights at birth and on Day 4 of lactation and the sex ratios on these days were not affected by the treatment, in any group.

At necropsy of females, the uterus of the female that failed to become pregnant in the 300 mg/kg treated group showed ballooning and accumulation of cloudy fluid. Histopathology of the uterus revealed lumen dilatation and cellular infiltration of neutrophils in the epithelium and endometrial stroma, with edema in the endometrial stroma. In the 1,000 mg/kg treated group, however, no abnormality was observed in the reproductive organs of the four females that failed to become pregnant, either, at the gross necropsy or in the histopathological examination of the ovary, while a moderate increase in atretic follicles was observed in one of the females. Including this case, there were no histopathological findings in the ovary related to doses of the compound (data not shown).

No abnormalities in the morphology or behavior of pups was noted in any group, except the following occasional cases in a single dam of the 1,000 mg/kg treated group: temporary cyanosis at birth and dilatation of renal pelvis at necropsy on Day 4 of lactation in one male pup.

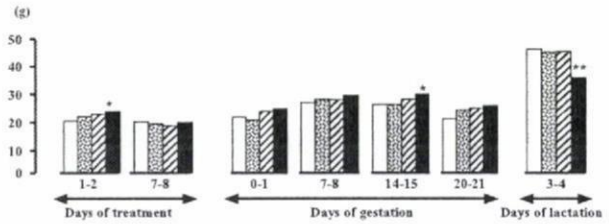

Fig. 6. Changes in food consumption of female rats treated orally with 4-methylbenzoic acid at dose levels of 0 (open column), 100 (dashed column), 300 (hatched column) or 1,000 mg/kg/day (closed column) in the reproduction/developmental toxicity screening test. Administration of the compound was started 2 weeks prior to mating, and was continued through mating period and gestation period until 3 days after delivery. Each value during the pre-mating treatment period represents the average for 13 females. That during the gestation and lactation periods represents the average for 9-13 dams. * and ** indicate significant difference from control at $p < 0.05$ and 0.01, respectively.

Table 5. Weights of testes and epididymides in rats treated orally with 4-methylbenzoic acid for 42-days in reproduction/developmental toxicity screening test

Dose (mg/kg)	0	100	300	1,000
Number of animals	13	13	13	13
Body weight (g)	527.9 ± 37.8	549.9 ± 42.8	541.6 ± 26.6	542.4 ± 30.7
Absolute weight				
Testes (g)	3.37 ± 0.24	3.29 ± 0.19	3.29 ± 0.22	3.31 ± 0.17
Epididymides (g)	1.28 ± 0.08	1.27 ± 0.08	1.24 ± 0.07	1.13 ± 0.09**
Relative weight (g/100 g)				
Testes	0.64 ± 0.07	0.60 ± 0.06	0.61 ± 0.04	0.61 ± 0.05
Epididymides	0.24 ± 0.03	0.23 ± 0.02	0.23 ± 0.01	0.21 ± 0.02**

Values represent average ± S.D.
**, significant difference from control at $p < 0.01$.

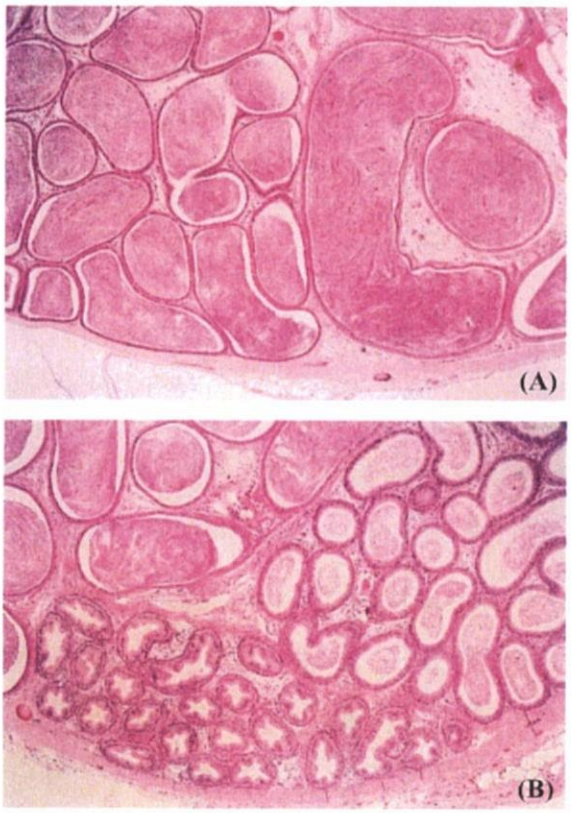

Fig. 7. Representative photographs of microscopic cross section of the cauda epididymis in male rats treated orally with 4-methylbenzoic acid at dose levels of 0 (A) or 1,000 mg/kg/day (B) in the reproduction study conducted under the OECD test guideline 421. Note that a few sperm are found in the lumen of the compound treated rats (B), and the epithelial height of the lumen becomes high. In contrast, the epithelial height of the lumen in the vehicle treated rats (A) becomes low by filling sperm in the lumen. Hematoxylin-eosin stain, x36.

DISCUSSION

Although the results obtained from the present studies are limited to short-term oral toxicity of 4-methylbenzoic acid, they indicate some toxicological properties of the compound. Namely, inconsistent effects of the compound on the epididymis indicate different toxicological potencies of the compound in male animals between the 28-day study and the reproduction/developmental study. While oral daily administration with 1,000 mg/kg of the compound reduced epididymal weight and caused oligo/azoospermia in the cauda epididymal lumen in the reproduction/developmental study, the same dose of the compound did not cause such effects in the 28-day study. Thus, the compound exerts adverse effects only on adult males when given for 42 days. Except that, repeated administration of 1,000 mg/kg of the compound led to similar results in males in both studies, such as a temporary salivation after dosing and no effects on body weight increase or food consumption. Therefore, the inconsistencies may be caused by differences in the timing or duration of administration.

The oligo/azoospermia seemed to develop at the distal part of the epididymis, since no abnormalities were observed in the caput epididymis and since there were normal lumens in the cauda epididymis. As reported by Robaire *et al.* (2006), spermatozoa enter the epididymis with testicular fluid and progress downward toward the vas deferens by smooth muscle contraction. In the reproduction/developmental study, the oligo/azoospermia developed where mature spermatozoa are stored. Spermatozoa are found in seminiferous tubules at the time of puberty, approximately 6 weeks of age in the rat, and it takes at least 10 or 15 days to reach the cauda epididymis (Sommer *et al.*, 1996; Robaire *et al.*, 2006). We have confirmed previously that spermatozoa are found in the cauda epididymis at 56 days of age at the youngest in the Sprague-Dawley derived inbred rats, which attain puberty at a comparable range of ages to Sprague-Dawley rats (Sato *et al.*, 2002). Since the compound was administered for 4 weeks in the 28-day study, from 5 weeks of age, the compound could influence epididymal spermatozoa for a short period. The storage period of spermatozoa in the cauda epididymis is estimated to be one week. In contrast, the compound was administered from 10 weeks of age in the reproduction/developmental study and could influence spermatozoa in all segments of the epididymis for 42 days. The inconsistent adverse effects of the compound on the cauda epididymis between the studies could be explained by either, the timing or the duration, of the administration.

Although the results obtained from the reproduction/developmental study seemed to suggest epididymal spermatozoa as the toxicological target of the compound, 4-methylbenzoic acid could influence spermatozoa function during their transit to the cauda epididymis. It has been reported that spermatozoa become functionally mature during transit to the distal part of the epididymis, while they are protected from oxidative stress and harmful xenobiotics by the blood-epididymis barrier and different types of antioxidant enzymes in each segment of the epididymis (Tengpwski *et al.*, 2007; Kim *et al.*, 2004; Robaire *et al.*, 2006). Therefore, detailed investigation of the effects of the compound on production, function and transition of spermatozoa is required.

Table 6. Histopathological findings of testis and epididymis in rats treated orally with 4-methylbenzoic acid for 42 days in the reproduction/ developmental toxicity screening test

Dose	0 mg/kg					100 mg/kg					300 mg/kg					1,000 mg/kg				
Grade	-	±	+	++	+++	-	±	+	++	+++	-	±	+	++	+++	-	±	+	++	+++
Testis	(13)					(13)					(13)					(13)				
Atrophy, focal, seminiferous tubule, bilateral	12	1	0	0	0	12	1	0	0	0	13	0	0	0	0	13	0	0	0	0
Multinucleated giant cell, seminiferous tubule	13	0	0	0	0	13	0	0	0	0	13	0	0	0	0	12	1	0	0	0
Epididymis	(13)					(13)					(13)					(13)				
A few number of sperm, lumen, cauda, bilateral	13	0	0	0	0	13	0	0	0	0	13	0	0	0	0	**0	11	2	0	0
																		##		
Cell debris, lumen, cauda, bilateral	12	1	0	0	0	13	0	0	0	0	13	0	0	0	0	8	5	0	0	0
Spermatid granuloma, cauda, unilateral	13	0	0	0	0	13	0	0	0	0	12	0	1	0	0	13	0	0	0	0

-, Negative; ±, Very slight; +, Slight; ++, Moderate; +++, Severe

** and ##, significant difference from control at $p < 0.01$ for incidence and grades of the findings, respectively.

Table 7. Mating performances and dam data of rats treated orally with 4-methylbenzoic acid in the reproduction/developmental toxicity screening test

Dose (mg/kg)	0	100	300	1,000
Mating performance				
Copulated pairs/Co-housed pairs (%)	13/13 (100)	13/13 (100)	12/13 (92.3)	13/13 (100)
Pregnant females/Copulated pairs (%)	13/13 (100)	13/13 (100)	11/12 (91.7)	9/13 (69.2)*
Pairing days until copulation [a]	3.0 ± 3.4	2.4 ± 1.3	2.4 ± 1.3	3.2 ± 3.3
Number of estrus revolved until copulation [a]	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.1 ± 0.3
Dam data				
Number of pregnant females with live pups	13	13	11	9
Gestation length in days [a]	22.3 ± 0.5	22.5 ± 0.5	22.3 ± 0.5	22.8 ± 0.4
Number of corpora lutea [a]	16.2 ± 1.3	15.7 ± 1.3	16.2 ± 1.5	15.9 ± 1.3
Number of implantation sites [a]	16.0 ± 1.3	15.4 ± 1.0	14.0 ± 2.4	12.1 ± 5.3
Implantation index [a, b]	99.1 ± 2.2	98.2 ± 3.5	86.5 ± 12.6**	75.9 ± 32.6*
Day 0 of lactation				
Number of pups born [a]	15.2 ± 1.4	14.1 ± 1.8	12.5 ± 2.1**	10.2 ± 5.1**
Delivery index [a, c]	94.7 ± 5.2	91.4 ± 8.5	90.1 ± 8.4	82.9 ± 19.5
Number of pups alive	14.3 ± 1.7	14.1 ± 1.8	12.3 ± 2.2	10.0 ± 5.0*
Birth index [a, d]	89.7 ± 10.4	91.4 ± 8.5	88.0 ± 9.1	81.5 ± 19.8
Live birth index [a, e]	94.8 ± 10.5	100 ± 0	97.6 ± 4.2	98.2 ± 3.7
Sex ratio [a, f]	54.1 ± 14.3	51.9 ± 12.8	46.6 ± 13.8	59.8 ± 17.7
Day 4 of lactation				
Number of pups alive [a]	14.3 ± 1.7	13.9 ± 1.8	12.3 ± 2.2	9.7 ± 5.3*
Viability index [a]	100 ± 0	99.0 ± 3.7	100 ± 0	88.1 ± 33.1
Sex ratio [a, f]	54.1 ± 14.3	52.3 ± 12.3	46.6 ± 13.8	54.4 ± 9.5
Pups weight on Day 0 of lactation (g/pup)				
Male [a]	6.8 ± 0.4	7.0 ± 0.5	7.0 ± 0.5	6.7 ± 0.3
Female [a]	6.5 ± 0.4	6.7 ± 0.6	6.6 ± 0.5	6.4 ± 0.5
Pups weight on Day 4 of lactation (g/pup)				
Male [a]	10.7 ± 1.0	11.2 ± 1.1	11.8 ± 1.2	11.1 ± 0.9
Female [a]	10.3 ± 1.0	10.7 ± 1.1	11.2 ± 1.3	10.5 ± 0.9

[a] Average ± S.D.; [b] (Number of implantation sites/Number of corpora lutea) × 100, %; [c] (Number of pups born/Number of implantation sites) × 100, %; [d] (Number of live pups on Day 0 of lactation/Number of implantation sites) × 100, %; [e] (Number of live pups on Day 0 of lactation/Number of pups born) × 100, %; [f] (Number of live male pups/Number of live pups) × 100, %
* and **, significant difference from control at $p < 0.05$ and 0.01, respectively.

The reproduction/developmental study revealed that the compound reduced fertility of animals within a relatively short period of dosing, since the four males that failed to impregnate in the 1,000 mg/kg treated group had copulated on Days 16, 19, 19 and 28 of treatment. Furthermore, the compound increased preimplantation loss and decreased implantation index at a lower dose level than that caused oligo/azoospermia. It is not clear whether the reduced fertility and the increase in the preimplantation loss are consequent effects of the compound on the epididymal spermatozoa or are effects on such as fertilization, early embryonic development or implantation, although the results obtained from the reproduction/developmental study indicate no adverse effects on female reproductive function until mating.

Because mating was done within the same dose groups

in the reproduction/developmental study, it was impossible to determine the target sex of the compound. The reproductive toxicities of several compounds related to 4-methylbenzoic acid have been studied. A feeding administration of *p*-nitrobenzoic acid (CAS No. 62-23-7) (NTP, 1994) and m-nitrobenzoic acid (CAS No. 121-92-6) has been found to reduce the number of offspring in continuous breeding studies in CD-1 mice (NTP/NIEHS, 1997). Crossover mating conducted in these studies revealed that female reproduction is more sensitive than male reproduction. On the other hand, 3-methylbenzoic acid (CAS No. 99-04-7) and 4-hydroxybenzoic acid (CAS No. 99-96-7) did not show any reproductive toxicity in studies conducted under a protocol similar to the reproduction/developmental study (Nagao *et al.*, 1997; Yamamoto *et al.*, 1999), although estrogenic potency of 4-hydroxybenzoic acid was observed in a uterotrophic assay (Lemini *et al.*, 1997). Detailed investigation, such as crossover mating, quantification and qualification of spermatozoa, may elucidate the characteristics of the reproductive toxicity of 4-methylbenzoic acid.

In the reproduction/developmental study, maternal body weight was reduced during the latter period of gestation at the dose levels of 300 mg/kg or more, whereas it did not affect female body weight, at any dose level, in the 28-day study. It is clear, however, that the decrease in the maternal body weight was not caused by the direct toxicity of the compound. It was caused by a small litter size resulting from a reduced number of implantations, as discussed above, since fetal weight in the uterus greatly contributes to maternal body weight during the latter period of gestation. Comparable or greater food consumption during this period and greater body weight after parturition in dams of these groups also indicate no adverse effects of the compound on the maternal animals. Decreases in food consumption and body weight gain during the lactation period in the 1,000 mg/kg treated group might be physiological changes due to a smaller demand of nutrition for the small litter size.

In the males, urine specific gravity was decreased in the 300 mg/kg or more treated groups. The change, however, was reflected the increase in urine volume due to increased water consumption, and did not accompany morphological alterations or functional impairments of their kidneys. Therefore, the change was judged as a physiological response. Except for the effects on the epididymis in the reproduction/development study, no systemic effect of the compounds was observed in the males of both studies.

In the females, a slight increase in food consumption was observed in the 1,000 mg/kg treated group at the beginning of dosing in both studies. In the 28-day study, increase in AST activity and decrease in serum total protein concentration were observed in the 1,000 mg/kg treated females. Since increase in AST activity has been reported as an effect of 3-methylbenzoic acid (CAS No. 99-04-7) in the study noted above (Yamamoto *et al.*, 1999), 4-methylbenzoic acid may slightly alter female AST activity. Thus, the compound affected females at the dose level of 1,000 mg/kg. However, all these changes did not accompany structural changes and changes in organ weights. Therefore, repeated dosing of the compound may affect females at the dose level of 1,000 mg/kg, but may not affect adversely, at any dose level.

From these results, the no-observed-effect-level (NOEL) for reproductive toxicity is considered to be 100 mg/kg, whereas 1,000 mg/kg did not show any effect on neonates. That for repeated dose toxicity is considered to be 300 mg/kg for male and female rats in the both studies, but toxic effects on the epididymis differed between the studies. Thus, 4-methylbenzoic acid has a potential for reproductive toxicity and deserves further study, taking the exposure state into consideration, as discussed above, since it has been reported that the reproduction/developmental study provides screening information but does not provide a complete characterization and evaluation of reproductive or developmental toxicity (Gelbke *et al.*, 2004).

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Why Public Health Agencies Cannot Depend on Good Laboratory Practices as a Criterion for Selecting Data: The Case of Bisphenol A

John Peterson Myers,[1] Frederick S. vom Saal,[2] Benson T. Akingbemi,[3] Koji Arizono,[4] Scott Belcher,[5] Theo Colborn,[6] Ibrahim Chahoud,[7] D. Andrew Crain,[8] Francesca Farabollini,[9] Louis J. Guillette Jr.,[10] Terry Hassold,[11] Shuk-mei Ho,[12] Patricia A. Hunt,[11] Taisen Iguchi,[13] Susan Jobling,[14] Jun Kanno,[15] Hans Laufer,[16] Michele Marcus,[17] John A. McLachlan,[18] Angel Nadal,[19] Jörg Oehlmann,[20] Nicolás Olea,[21] Paola Palanza,[22] Stefano Parmigiani,[22] Beverly S. Rubin,[23] Gilbert Schoenfelder,[24] Carlos Sonnenschein,[23] Ana M. Soto,[23] Chris E. Talsness,[25] Julia A. Taylor,[2] Laura N. Vandenberg,[23] John G. Vandenbergh,[26] Sarah Vogel,[27] Cheryl S. Watson,[28] Wade V. Welshons,[29] and R. Thomas Zoeller[30]

[1]Environmental Health Sciences, Charlottesville, Virginia, USA; [2]Division of Biological Sciences, University of Missouri, Columbia, Missouri, USA; [3]Department of Anatomy, Physiology and Pharmacology, College of Veterinary Medicine, Auburn University, Auburn, Alabama, USA; [4]Faculty of Environmental and Symbiotic Science, Prefectural University of Kumamoto, Tsukide, Kumamoto, Japan; [5]Department of Pharmacology and Cell Biophysics, Center for Environmental Genetics, University of Cincinnati, Cincinnati, Ohio, USA; [6]The Endocrine Disruption Exchange, Paonia, Colorado, USA; [7]Institut für Klinische Pharmakologie und Toxikologie Charité, Universitätsmedizin Berlin, Campus Benjamin Franklin, Berlin, Germany; [8]Department of Biology, Maryville College, Maryville, Tennessee, USA; [9]Dipartimento di Fisiologia, Università di Siena, Siena, Italy; [10]Department of Zoology, University of Florida, Gainesville, Florida, USA; [11]School of Molecular Biosciences, Washington State University, Pullman, Washington, USA; [12]Department of Environmental Health, University of Cincinnati, Cincinnati, Ohio, USA; [13]National Institutes of Natural Science, Okazaki Institute for Integrative Bioscience, Bioenvironmental Science, Okazaki, Japan; [14]Department of Biological Sciences, Brunel University, Uxbridge, United Kingdom; [15]Division of Cellular and Molecular Toxicology, National Institute of Health Sciences, Tokyo, Japan; [16]Department of Molecular and Cell Biology, University of Connecticut, Storrs, Connecticut, USA; [17]Department of Epidemiology, Rollins School of Public Health, Emory University, Atlanta, Georgia, USA; [18]Center for Bioenvironmental Research, Tulane and Xavier Universities, New Orleans, Louisiana, USA; [19]Instituto de Bioingeniería and CIBERDEM, Universidad Miguel Hernández de Elche, Alicante, Spain; [20]Goethe University Frankfurt am Main, Department Aquatic Ecotoxicology, Frankfurt, Germany; [21]Hospital Clínico, CIBERESP, University of Granada, Granada, Spain; [22]Dipartimento di Biologia Evolutiva e Funzionale, Universita' di Parma, Parma, Italy; [23]Tufts Medical School, Boston, Massachusetts, USA; [24]Institute of Pharmacology and Toxicology, University of Wuerzburg, Wuerzburg Germany; [25]Charité University Medical School Berlin, Berlin, Germany; [26]Department of Biology, North Carolina State University, Raleigh, North Carolina, USA; [27]Chemical Heritage Foundation, Philadelphia, Pennsylvania, USA; [28]Biochemistry and Molecular Biology, University of Texas Medical Branch, Galveston, Texas, USA; [29]Department of Biomedical Sciences, University of Missouri, Columbia, Missouri, USA; [30]Biology Department, University of Massachusetts, Amherst, Massachusetts, USA

Background: In their safety evaluations of bisphenol A (BPA), the U.S. Food and Drug Administration (FDA) and a counterpart in Europe, the European Food Safety Authority (EFSA), have given special prominence to two industry-funded studies that adhered to standards defined by Good Laboratory Practices (GLP). These same agencies have given much less weight in risk assessments to a large number of independently replicated non-GLP studies conducted with government funding by the leading experts in various fields of science from around the world.

Objectives: We reviewed differences between industry-funded GLP studies of BPA conducted by commercial laboratories for regulatory purposes and non-GLP studies conducted in academic and government laboratories to identify hazards and molecular mechanisms mediating adverse effects. We examined the methods and results in the GLP studies that were pivotal in the draft decision of the U.S. FDA declaring BPA safe in relation to findings from studies that were competitive for U.S. National Institutes of Health (NIH) funding, peer-reviewed for publication in leading journals, subject to independent replication, but rejected by the U.S. FDA for regulatory purposes.

Discussion: Although the U.S. FDA and EFSA have deemed two industry-funded GLP studies of BPA to be superior to hundreds of studies funded by the U.S. NIH and NIH counterparts in other countries, the GLP studies on which the agencies based their decisions have serious conceptual and methodologic flaws. In addition, the U.S. FDA and EFSA have mistakenly assumed that GLP yields valid and reliable scientific findings (i.e., "good science"). Their rationale for favoring GLP studies over hundreds of publically funded studies ignores the central factor in determining the reliability and validity of scientific findings, namely, independent replication, and use of the most appropriate and sensitive state-of-the-art assays, neither of which is an expectation of industry-funded GLP research.

Conclusions: Public health decisions should be based on studies using appropriate protocols with appropriate controls and the most sensitive assays, not GLP. Relevant NIH-funded research using state-of-the-art techniques should play a prominent role in safety evaluations of chemicals.

Key words: bisphenol A, endocrine disruptors, FDA, Food and Drug Administration, GLP, good laboratory practices, low-dose, nonmonotonic, positive control. *Environ Health Perspect* 117:309–315 (2009). doi:10.1289/ehp.0800173 available via *http://dx.doi.org/* [Online 22 October 2008]

Regulatory agencies in the United States and the European Union (EU) have justified the decision to declare the estrogenic chemical bisphenol A (BPA) safe at current levels of human exposure based on a few studies conducted using Good Laboratory Practices (GLP). In contrast, these agencies have rejected for consideration in their risk assessment of BPA hundreds of laboratory animal and mechanistic cell culture studies conducted by academic and government scientists reporting harm at very low doses of BPA. These studies were rejected primarily because they were not conducted using GLP. We suggest that decisions based on this logic are misguided and will result in continued risk to public health from exposure to BPA, as well as other manmade chemicals.

GLP is a federal rule for conducting research on the health effects or safety testing of drugs or chemicals submitted by private research companies for regulatory purposes. The GLP outlines basic guidelines for conducting scientific research, including the care and feeding of laboratory animals, standards for facility maintenance, calibration and care of equipment, personnel requirements, inspections, study protocols, and collection and storage of raw data (Goldman 1988). These regulations were developed in response to widespread misconduct by private research companies; this misconduct was possible because their data usually do not go through the rigorous, multistage scientific review that is normal for academic data funded by federal agencies and published in the peer-reviewed literature. The lack of these safeguards from academic science had enabled fraud. The U.S. Food and Drug

Address correspondence to J.P. Myers, Environmental Health Sciences, 421 Park St., Charlottesville, VA 22902 USA. Telephone: (434) 220-0348. Fax: (434) 220-0347. E-mail: jpmyers@ehsic.org

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Administration (U.S. FDA) first issued rules for GLP in 1978 after a 2-year federal investigation into sloppy laboratory practices of a number of private research companies (Lublin 1978; Markowitz and Rosner 2002). What began as serious concerns about poor quality research expanded into a criminal investigation of Industrial Bio-Test (IBT), one of the largest private laboratories at the time and a subsidiary of Nalco Chemical Company. In response to the federal investigation, the U.S. Environmental Protection Agency (EPA) demanded that 235 chemical companies reexamine the > 4,000 tests conducted by the laboratory. In 1983, three men from IBT were found guilty of deliberating doctoring data and were sentenced to prison (Lublin 1978; Markowitz and Rosner 2002). The fraudulent practices of IBT brought into question 15% of the pesticides approved for use in the United States. That same year, the U.S. EPA issued similar GLP rules for regulatory testing.

Both the U.S. FDA (2008a) and European Food Safety Authority (ESFA 2006) have recently published documents demonstrating that their decision to continue to declare BPA safe at current exposure levels was based primarily on the results of a few industry-funded studies that followed GLP guidelines. These decisions stand in stark contrast to the decisions concerning the potential risks to human health reached by a panel of 38 experts at a U.S. National Institutes of Health (NIH)-sponsored conference, who published The Chapel Hill Consensus Statement (vom Saal et al. 2007), as well as five review articles (Crain et al. 2007; Keri et al. 2007; Richter et al. 2007a; Vandenberg et al. 2007a; Wetherill et al. 2007). These peer-reviewed articles covered approximately 700 articles concerning BPA and represented a comprehensive review of the literature as of the end of 2006. In addition, the U.S. FDA draft decision contradicted the conclusions reached by the National Toxicology Program (NTP), which had spent 2 years investigating this question (NTP 2008). An important role of the NTP is to advise the U.S. FDA about the science relating to toxic chemicals in food, but in an unusual move, the U.S. FDA chose to release its draft report before the release of the final report on BPA by the NTP and without indicating who at the U.S. FDA was involved in preparing the draft report (U.S. FDA 2008b). At a hearing on 16 September 2008 regarding the draft report on BPA, the U.S. FDA announced that their goal was to have a subcommittee of the U.S. FDA Science Board complete a review of the draft decision by the end of October 2008. This would presumably also involve review by the subcommittee members of the approximately 1,000 articles relating to BPA.

We believe that the methods employed in chemical industry–sponsored GLP studies are incapable of detecting low-dose endocrine-disrupting effects of BPA and other hormonally active chemicals. Detecting endocrine-disrupting effects at low doses of chemicals such as BPA requires sophisticated and modern assays and analyses that have been developed in advanced, usually federally funded laboratories over the past decade. This is especially apparent when one examines what is now known about functional effects of BPA on a wide range of end points (Richter et al. 2007a; Welshons et al. 2006; Wetherill et al. 2007). These end points include those mediated by recently discovered estrogen response pathways initiated in human and animal cell membranes (nonclassical or alternative estrogen response mechanisms), which multiple laboratories have shown to be equally sensitive to BPA and estradiol in terms of activating effects in human and animal cells at low picomolar through low nanomolar concentrations (Alonso-Magdalena et al. 2008; Wetherill et al. 2007; Wozniak et al. 2005; Zsarnovszky et al. 2005).

The effects of BPA documented in these studies include a diverse array for which there are no data from GLP studies because the end points have not been examined: altered metabolism related to metabolic syndrome (Alonso-Magdalena et al. 2005, 2006, 2008; Ropero et al. 2008); altered adiponectin secretion (Hugo et al. 2008), which is a condition predicting heart disease and type 2 diabetes (Lang et al. 2008); altered epigenetic programming leading to precancerous lesions of the prostate (Ho et al. 2006); differential growth patterns in the developing prostate (Timms et al. 2005); abnormal growth, gene expression, and precancerous lesions of the mammary glands (Soto et al. 2008); and adverse effects on the female reproductive system, including uterine fibroids, paraovarian cysts, and chromosomal abnormalities in oocytes (Newbold et al. 2007; Susiarjo et al. 2007). There is also a large literature on neuroanatomic, neurochemical, and behavioral abnormalities caused by low doses of BPA (Leranth et al. 2008; Richter et al. 2007a), which also are not capable of being detected by current GLP studies conducted for regulatory purposes because of their out-of-date assays.

The approaches used by academic and government scientists to study the potential health hazards of BPA contrast sharply with those still used by the chemical industry that are relied on by regulatory agencies in the United States and Europe, including the two studies identified by both the U.S. FDA and European Food Safety Authority (EFSA) as central to the decision to declare BPA safe at current human exposure levels (Tyl et al. 2002, 2008a). By using outdated and insensitive assays that were supposed to have been replaced by a new battery of screens and tests by 2000 [as mandated by the U.S. Congress in 1996 in the Food Quality Protection Act (1996), but which has, as yet, still not occurred], these studies conducted using GLP fail to find any adverse effects.

Reliability and Validity

Reliability and validity are separate issues, although in the experimental research described here, validity and reliability basically refer to research that is credible. Golafshani (2003) noted that "reliability" refers to the extent to which results are consistent over time and are an accurate representation of the total population under study. Of central importance is that the results of a study must be reproduced under a similar methodology to be considered to be reliable. "Validity" refers to whether the research measures what it was intended to measure, and valid findings are considered to be true. In other words, reliability is determined by whether the results are replicable, whereas validity is assessed by whether the methods used result in finding the truth as a result of the investigator actually measuring what the study intended to measure.

Use of GLP in Regulatory Decision Making

Despite strong evidence of aberrations caused by low doses of BPA in animals exposed during fetal and neonatal life in studies conducted by the world's leading academic and government experts in the fields of endocrine disruption, endocrinology, neurobiology, reproductive biology, genetics, and metabolism, a relatively small number of studies reporting no adverse effects at low doses of BPA have continued to be promoted by the chemical industry and used by regulatory agencies (e.g., Ashby et al. 1999; Cagen et al. 1999; Tyl et al. 2002, 2008a). According to the U.S. FDA, these are accepted because they used GLP (U.S. EPA 2008), with the implication that studies not employing GLP are not reliable or valid (U.S. FDA 2008a).

GLP does not guarantee reliability or validity of scientific results. Unfortunately, although GLP creates the semblance of reliable and valid science, it actually offers no such guarantee. GLP specifies nothing about the quality of the research design, the skills of the technicians, the sensitivity of the assays, or whether the methods employed are current or out-of-date. (All of the above are central issues in the review of a grant proposal by an NIH panel.) GLP simply indicates that the laboratory technicians/scientists performing experiments follow highly detailed U.S. EPA requirements [or in the EU, Organization for Economic Co-operation and Development (OECD) requirements] for record keeping, including details of the conduct of the

experiment and archiving relevant biological and chemical materials (U.S. EPA 2008).

These record-keeping procedures in GLP were instituted because of widespread misconduct being committed by commercial testing laboratories (described above). These fraudulent results were possible because contract laboratory studies used in the regulatory process are rarely subject to the checks and balances that peer-reviewed, replicated scientific findings undergo. Without that acid test of reliability (replication by other independent scientists), other procedures were needed. Hence GLP was implemented, despite its severe limitations.

NIH-funded research subject to more stringent reviews than GLP. Although few NIH-funded investigators adhere to GLP-mandated record keeping, the procedures of GLP are actually surpassed by the procedures required for NIH-funded science published in peer-reviewed journals. NIH-funded studies pass through three phases of peer review that are far more challenging than GLP requirements. First, the principal scientists must have demonstrated competence to conduct the research, and experimental methods, assays, and laboratory environment must involve use of state-of-the-art techniques to be competitive for NIH funding. Second, results are published in peer-reviewed journals, with detailed evaluations by independent experts examining all aspects of the study. And third, the findings are challenged by independent efforts to replicate; for example, the initial findings concerning the stimulating effects of estrogenic chemicals on the mouse prostate (Nagel et al. 1997; vom Saal et al. 1997) were independently replicated and extended by Gupta (2000), which led to an editorial identifying "initial results confirmed" (Sheehan 2000).

Typically, within a laboratory, interesting findings are also followed by subsequent publications extending the prior findings; examples include the findings of BPA effects on β cells in the mouse pancreas (Alonso-Magdalena et al. 2005, 2006, 2008) and the effects of estrogenic chemicals and drugs on the developing mouse prostate that followed earlier findings (described above) from this same group (Timms et al. 2005; Richter et al. 2007b). In particular, independent replication by competent, respected scientists is the main criterion of acceptance of the findings as having been demonstrated to be reliable and having been validated by virtue of coming to the same conclusion using a variety of sophisticated techniques in multiple publications.

An important criticism of the approach taken by the U.S. FDA in its assessment of the now approximately 1,000 articles on BPA is that it appears to have made no attempt to connect the dots between replicated studies; instead, the U.S. FDA appears to have assessed each study without regard to whether it had been confirmed by other studies.

Thus, collectively, many phases used to verify the reliability and validity of NIH-funded published research have been completely ignored by the U.S. FDA, whereas industry-funded GLP research is rarely, if ever, subject to these central requirements and yet is accepted by regulatory agencies as reliable and valid.

The U.S. FDA's misguided gold standard. In this light, the U.S. FDA's reliance upon GLP as the gold standard is scientifically misguided. Furthermore, U.S. FDA administrators are ignoring published critiques of the GLP studies it considers reliable and valid, such as the study by Tyl et al. (2002) and two coordinated studies conducted at the same time by Ashby et al. (1999) and Cagen et al. (1999). Each was an industry-funded study conducted using GLP. Each was harshly criticized in peer-reviewed publications by academic scientists and government panels [Center for the Evaluation of Risks to Human Reproduction (CERHR) 2007; NTP 2001; vom Saal and Hughes 2005; vom Saal and Welshons 2006]. Yet, the U.S. FDA and EFSA panels still assert that these studies represent the gold standard in toxicologic research.

Specifically, the studies of Cagen et al. (1999) and Ashby et al. (1999) were recently rejected by the NTP CERHR panel on BPA as unusable for consideration in its evaluation of the health hazards posed by BPA (CERHR 2007). Both the Ashby et al. (1999) and Cagen et al. (1999) studies reported finding no effect of their positive control [the estrogenic drug diethylstilbestrol (DES)] on any outcome, although these failures were not acknowledged by the authors in either article. In experimental science, the failure of a positive control to show an effect indicates the experiment failed, which is the conclusion reached by the CERHR panel (CERHR 2007).

The Tyl et al. 2002 study, which the U.S. FDA still accepts as a major study for determination of the safety of BPA (U.S. FDA 2008a, 2008b), was criticized by an NTP panel that met in 2000 to examine the low-dose issue (NTP 2001), as well as in subsequent publications (vom Saal and Hughes 2005; vom Saal and Welshons 2006), for using an insensitive rat (the CD-SD rat) that requires extremely high doses (≥ 50 μg/kg/day) of the potent estrogenic drug ethinylestradiol to show effects such as those examined in the study by Tyl et al. (2002). This dose of ethinylestradiol is > 100 times higher than the approximately 0.3 μg/kg/day used by women in oral contraceptives. The fact that Tyl et al. (2002) adhered to GLP did not protect them from using insensitive animals. This led the NTP (2001) to state:

> Because of clear species and strain differences in sensitivity, animal model selection should be based on responsiveness to endocrine-active agents of concern (i.e., responsive to positive controls), not on convenience and familiarity.

Thus, when reviewed by other scientists, three prior major GLP studies of BPA have been found to be so flawed as to be useless for guiding regulatory agencies in decision making. A new GLP study has now been published by Tyl et al. (2008a). Close examination of this study also reveals fatal flaws which render it useless for regulatory purposes, even though it conforms to GLP.

Examples of Flaws Ignored by the U.S. FDA and EFSA in a Recent GLP Study of BPA

In summary, the flaws in Tyl et al. (2008a) are as follows:

- The high dose required for the positive control (estradiol) to cause an effect means the system used by Tyl et al. (2008a), at least in her laboratory, is relatively insensitive to exogenous estrogens and thus inappropriate for studying low-dose effects of estrogenic compounds such as BPA. The lack of response to low doses of estradiol or BPA in the Tyl laboratory is puzzling, in that the strain of mice used in these experiments (the CD-1 mouse) has been reported in > 20 other peer-reviewed publications to show adverse effects in response to very low doses of BPA (vom Saal 2008), as well as many other studies showing low-dose effects in response to the natural hormone estradiol, the estrogenic drugs ethinylestradiol and DES, and to other estrogenic chemicals.
- Tyl et al. (2008a) used insensitive, out-of-date protocols and assays that are incapable of finding many of the adverse effects reported by more sophisticated studies conducted by independent NIH-funded scientists as well as scientists funded by government agencies in other countries.
- In the specific case of testing for changes in prostate weight, Tyl et al. (2008a) reported an abnormally high prostate weight for control animals that exceeds by > 70% the prostate weights reported by other studies for animals of the same strain and similar age (e.g., Gupta 2000; Ruhlen et al. 2008). This suggests that the dissection procedures for the prostate in the Tyl laboratory included other nonprostatic tissues in the weight measurements, rendering them unusable for studying weight changes in the prostate in response to BPA or estradiol; neither chemical showed any effect on the selected end points, which directly contradicts other findings concerning opposite effects of low and high doses of estrogen on the prostate (Putz et al. 2001; Timms et al. 2005; vom Saal et al. 1997).

Aberrant insensitivity of CD-1 mouse to estrogens. Tyl et al. (2008a) used estradiol as a positive control. It was fed to female mice before and during pregnancy and lactation at 80–220 μg/kg/day; after weaning, estradiol was fed to offspring at doses of 80–100 μg/kg/day. Estradiol was used as a positive control because BPA is a man-made endocrine-disrupting estrogenic chemical.

Many published findings reporting effects of very low doses of positive control estrogens and BPA in CD-1 mice demonstrate that the CD-1 mouse was somehow rendered insensitive in the test system used by Tyl et al. (2008a). The fact that a dose of 100–200 μg/kg/day estradiol was necessary to show an effect of the positive control predicts that Tyl et al. (2008a) should not detect effects of BPA < 10–100 mg/kg/day, far above the low-dose range relevant to human exposures that was supposedly of interest.

For nuclear estrogen receptor–mediated effects via regulation of gene activity (nuclear estrogen receptors are transcription factors whose activity is regulated by binding to estrogen), prior studies have typically shown a 1,000-fold lower activity for BPA relative to estradiol or potent estrogenic drugs, including DES and ethinylestradiol. For example, Richter et al. (2007b) reported an increase in androgen receptor gene activity to estradiol at 1 pM (0.28 pg/mL) in fetal CD-1 mouse prostatic mesenchyme cells in primary culture, and the same response was found for BPA at 1,000 pM (228 pg/mL); the *in vitro* response to estradiol was predicted by the response of the prostate to increasing free serum estradiol from 0.2 to 0.3 pg/mL in male mouse fetuses via estradiol administration to the mother (vom Saal et al. 1997). Other research showed that a significant effect on development of the male reproductive system in CF-1 mice occurred at a maternal dose of 0.002 μg/kg/day ethinylestradiol (Thayer et al. 2001), similar to effects observed with 2–20 μg/kg/day BPA (vom Saal et al. 1998). The research of Honma et al. (2002) showed accelerated puberty in CD-1 (ICR) mice at a DES dose of 0.02 μg/kg/day (the positive control), and the same response to BPA occurred at 20 μg/kg/day, again revealing a 1,000-fold difference between the positive control estrogen and BPA.

There are many other examples of findings where a higher dose of BPA was required to cause the same effect as the positive control estrogen (estradiol, ethinylestradiol, or DES) in studies where the effects were mediated by the classical nuclear estrogen receptors, in contrast to the more recently discovered rapid signaling estrogen response system where BPA and these positive control estrogens have equal potency, as described above. In summary, CD-1 mice have been used by a large number of academic and government investigators and have been reported in peer-reviewed publications to be sensitive to positive control estrogens within the range of human sensitivity based on *in vivo* and *in vitro* studies via the classical estrogen receptor α–mediated response mechanism. The CD-1 mouse is the animal model that has been used by the U.S. National Institute of Environmental Health Sciences (NIEHS) for decades, because it is considered the best animal model for predicting the effects of developmental exposure to estrogen in humans (Newbold 1995; Newbold et al. 2007).

The failure of traditional toxicologic studies conducted by Tyl et al. (2008a, 2008b) to detect the wide range of adverse effects of even relatively high doses of BPA or of low doses of estradiol that have been reported in numerous studies by academic and government scientists provides evidence that the GLP protocols established long ago by regulatory agencies to determine the toxicity of chemicals are inappropriate for detecting the endocrine-disrupting activities of chemicals such as BPA. Indeed, this was the premise of the congressional mandate in the Food Quality Protection Act (1996) for the U.S. EPA to establish a new set of assays for endocrine-disrupting chemicals, although this process has been systematically delayed and is > 8 years behind the congressionally mandated date of 2000 to have these new assays validated.

Citing Tyl et al. (2008a), the EFSA report on BPA (EFSA 2006) stated that "the positive control substance, 17β-estradiol, resulted in reproductive and developmental toxicity." This report failed to acknowledge that only a very high dose of the positive control was sufficient to elicit effects and that this meant that the experiments conducted in the Tyl laboratory were for some reason very insensitive to any estrogen and thus inappropriate for use in a study to examine low-dose estrogenic effects of BPA.

Based on the preliminary report released by the U.S. FDA regarding BPA (U.S. FDA 2008a), it appears that the U.S. FDA has followed the lead of the EFSA in its lack of understanding of the importance of the dose of the positive control estrogen required to cause adverse effects. The consequence is that the U.S. FDA has relied primarily on the study of Tyl et al. (2008a, 2008b), with the result that the U.S. FDA has assured Americans that BPA is safe at current human exposure levels.

Several factors might account for the insensitivity of the CD-1 mouse in the Tyl et al. studies (2008a, 2008b) conducted at Research Triangle Institute (RTI), a testing facility that conducted these (as well as previous) studies funded by the American Chemistry Council. One possibility is that the diet used in these studies may have interfered with the results. The feed used by Tyl et al. (2008a) in this experiment (Purina 5002) has been shown by others to interfere with responses to exogenous estrogenic chemicals, blocking adverse effects documented on other diets. For example, a number of years ago, Thigpen et al. (2003) at the NIEHS recommended against the use of Purina 5002 in studies of endocrine-disrupting chemicals. Tyl et al. (2008a) measured some specific phytoestrogens in Purina 5002 feed by chemical analysis; however, in a report on NIH-sponsored meetings on this subject, Heindel and vom Saal (2008) pointed out that this is an insufficient control for total dietary estrogenic contaminants that can disrupt studies involving the effects of estrogenic chemicals.

A second possibility is that there are strain differences in sensitivity developed in the CD-1 mouse sold by the various Charles River Laboratories located in different regions. We consider this unlikely, because most laboratories regularly replace their CD-1 mouse breeder stock from Charles River Laboratories, and practices there make it unlikely that the sensitivity of this outbred stock to estrogens has changed dramatically over a very short period of time. Also, because RTI, where the Tyl studies were conducted, is very near the laboratories of the NIEHS, it is likely that the CD-1 mice used by these two programs were purchased from the same breeding facility.

Use of insensitive, out-of-date protocols and assays. Another serious concern about the two recent studies by Tyl et al. (2008a, 2008b) is the experimental approach used, thus raising questions about the validity of the studies. The study design used by Tyl et al. (2008a, 2008b) has been superseded by advances in both experimental design and analytical tools developed by NIH-funded scientists (and their counterparts in Europe and Asia) since the mid-1990s. The methods used by Tyl et al., primarily wet weight changes of tissues, gross histologic changes, and developmental landmarks such as vaginal opening, were established procedures by the 1950s. Thus, a major limitation of the Tyl studies is the failure to measure more meaningful and sensitive end points in order to detect the effects of low-dose BPA exposure, which are often not macroscopic in nature. Indeed, in 2001, the director of the reproductive division of the National Health and Environmental Effects Research Laboratory at the U.S. EPA stated that the inconclusive results concerning effects of BPA on reproductive toxicology can only be solved by understanding the mechanisms (Triendl 2001). With current GLP standards it is not possible to study mechanisms because they still rely on out-of-date assays.

As one example of a comparison between the approach by Tyl et al. (2008a) and independent government-funded academic scientists, extensive research has been conducted by Soto et al. (2008) and by other independent academic and government scientists

describing effects of exposure of female mice and rats to very low doses of BPA during perinatal development on the mammary glands (Jenkins et al. 2009). Although Tyl et al. (2008a) reported no low-dose effects of BPA on the mammary glands using conventional histologic analysis, there have been consistent findings of adverse effects of low doses of BPA from studies that used more sophisticated and sensitive analysis of whole mounted mammary glands to facilitate detection of microscopic lesions, coupled with immunostaining for regulatory proteins as well as techniques for determination of aberrant gene expression associated with progression to cancer. These peer-reviewed studies have reported detecting changes during embryonic development of mammary glands as well as abnormalities detected during adolescence through adulthood that are indicative of mammary gland cancer as well as other developmental abnormalities (Colerangle and Roy 1997; Durando et al. 2007; Jenkins et al. 2009; LaPensee et al. 2008; Markey et al. 2001, 2005; Moral et al. 2008; Munoz-de-Toro et al. 2005; Murray et al. 2007; Nikaido et al. 2004; Vandenberg et al. 2006, 2007b; Wadia et al. 2007).

Similar to the findings for the mammary gland, Ogura et al. (2007) reported that if tissues were analyzed by conventional histologic methods (staining with hematoxalin and eosin), prenatal exposure to low doses of BPA or DES showed no effects on prostate development, whereas if the sections were analyzed using antibodies that identified basal cells and basal cell squamous metaplasia, then significant effects were revealed. Squamous metaplasia of basal cells indicates abnormal proliferation and function of the prostate stem cell population that is thought to transform into neoplastic cells; Ho et al. (2006) reported that neonatal exposure to very low doses of BPA caused 100% of male rats to develop high-grade prostatic intraepithelial neoplastic lesions later in life. All of these studies were rejected by the U.S. FDA as not adequate for making regulatory decisions about the safety of BPA. Instead, the U.S. FDA relied upon Tyl et al. (2008a), even though the study used techniques that Ogura et al. (2007) showed lacked the sensitivity of 21st century experimental approaches.

Although findings regarding changes in brain structure, brain chemistry, and behavior represent the largest portion of the literature on low-dose BPA, Tyl et al. (2008a) did not examine any neurobehavioral end points. The NTP (2008) and the NIEHS conference consensus reports (vom Saal et al. 2007) both indicated concern about neurobehavioral effects of low doses of BPA. Thus, the absence of studies that included neurobehavioral end points is a glaring omission of Tyl et al. (2008a, 2008b).

Flawed prostate dissection. Data presented by Tyl et al. (2008a) raise questions about the adequacy of techniques used in their BPA studies. Specifically, Tyl et al. (2008a) reported that the prostate in 3.5-month-old control male CD-1 mice weighed > 70 mg [see Table 3 in Tyl et al. (2008a) for data on F_1 retained males]. This average control weight contrasts sharply with those reported from other laboratories. Specifically, the weight of the prostate in 2- to 3-month-old CD-1 mice using the dissection technique based on both Ruhlen et al. (2008) and Gupta (2000) and at the NIEHS (Newbold RR, personal communication) is about 40 mg. Several studies have reported that prenatal exposure to very low doses of BPA and positive control estrogens increased prostate size, prostatic androgen receptors, and prostate androgen receptor gene activity (Gupta 2000; Richter et al. 2007b; Thayer et al. 2001; Timms et al. 2005; vom Saal et al. 1997), but the enlarged prostate of experimental animals exposed to BPA in these laboratories weighed less than the prostates in the control animals of Tyl et al. (2008a). This raises serious questions about the procedures and/or animals used by Tyl et al. The weight of prostate reported by Tyl et al. (2008a) suggests that the technique used for dissecting the prostate resulted in nonprostatic tissue being weighed along with prostate. The seminal vesicle, coagulating gland, and dorsolateral prostate all merge together where the ejaculatory ducts enter the urethra, and there are also fat deposits on the prostate. This poses a challenge for those without proper training in distinguishing these different tissues during dissection in mice.

Alternatively, as male rodents age, they are prone to develop prostatitis. Although this inflammatory disease leads to an increase in prostate size and could thus account for the very large prostate weights reported by Tyl et al. (2008a), anyone familiar with the appearance of prostatitis would detect this abnormality upon histologic examination, which Tyl et al (2008a) supposedly conducted. Also, prostatitis is rare in young-adult mice or rats (Cowin et al. 2008), and the size of the prostates in the Tyl et al. (2008a) study were similar to those for middle-aged and old male mice.

The findings regarding effects of BPA on the prostate presented by Tyl et al. (2008a) are thus suspect and cannot be used as evidence that other earlier studies (Gupta 2000; Timms et al. 2005; vom Saal et al. 1997) are not replicable. Given these problems in prostate weight measurements, it is not surprising that even very high doses of BPA or estradiol reported by Tyl et al. (2008a) had no effect on the prostate, in sharp contrast to other studies that showed stimulation of the prostate at low doses of estrogen and inhibition at high doses (Putz et al. 2001; Timms et al. 2005).

In addition to the problem associated with the high prostate weight reported by Tyl et al. (2008a), in a separate measurement the authors combined the anterior prostate (coagulating gland) and seminal vesicle, presenting these two organs as one combined outcome measure. This is wrong and misleading. The coagulating glands emerge as the anterior ducts of the prostate from the dorsocranial region of the urogenital sinus, whereas the seminal vesicles bud from the proximal region of the Wolffian ducts. Elevated estrogen is associated with an increase in prostate size associated with an increase in prostate androgen receptors, whereas a decrease in seminal vesicle size is associated with a reduction in 5α-reductase, an enzyme that converts testosterone to the more potent androgen 5α-dihydrotestosterone (Nonneman et al. 1992). Low doses of BPA have been shown to decrease the size of organs that differentiate from the embryonic Wolffian ducts (epididymides and seminal vesicles) while increasing the size of regions of the prostate that develop from the urogenital sinus (vom Saal et al. 1998). Combining these different organs (it is technically not difficult to separate them) was thus inappropriate because they develop from different embryonic tissues that show markedly different responses to estrogenic chemicals during development. In fact, Ogura et al. (2007) reported that the anterior prostate (coagulating glands) showed the greatest expression of ER-α, and also showed the most pronounced indication of basal cell squamous metaplasia in response to developmental exposure to low doses of DES and BPA relative to other regions of the prostate.

Conclusions

Because the control data of Tyl et al. (2008a) were not consistent with the prior published literature for prostate weight of young-adult CD-1 male mice and because their methods were inappropriate for revealing an extensive body of adverse effects detected using more sophisticated approaches, we deem the findings by Tyl et al. to be invalid. Hundreds of studies show adverse effects of BPA in animals, with many conducted at concentrations equivalent to current human levels of BPA exposure; thus, it is unlikely that academic scientists would bother to replicate the outdated approaches used by Tyl et al. (2008a, 2008b). This lack of replication is typical of GLP studies, which tend to involve unnecessarily large numbers of animals [Tyl et al. (2002) used > 8,000 rats], and reliability appears to be accepted because of the numbers of animals that were used. Although using excessive numbers of animals is accepted as good science by the U.S. FDA, the use of arbitrarily large numbers of animals per group (> 20 animals per treatment group is common) actually violates guidelines in the NIH *Guide for the*

Care and Use of Laboratory Animals (Institute of Laboratory Animal Research 1996) that govern research conducted by academic and government scientists. For research with animals to be approved by any university animal care and use committee, group sizes must be based on power analysis conducted using historic data. Based on this criterion in the NIH Guide, all of the studies by Tyl et al. were significantly over powered and thus in direct violation of federal guidelines for conducting animal research, a fact about which U.S. FDA regulators seem unaware.

Each of the four main industry-funded GLP studies of BPA (Ashby et al. 1999; Cagen et al. 1999; Tyl et al. 2008a, 2008b) is flawed and not appropriate for use in setting health standards. Clearly, meeting GLP standards is not a guarantee of reliable or valid science. It is of great concern that the U.S. and EU regulatory communities are willing to accept these industry-funded, antiquated, and flawed studies as proof of the safety of BPA while rejecting as invalid for regulatory purposes the findings from a very large number of academic and government investigators using 21st-century scientific approaches. The basis for these decisions by U.S. and EU regulatory agencies should be thoroughly investigated, particularly since the NTP (2008) concluded that BPA exposure to human infants was in the range shown to cause harm in experimental animals and since both the Canadian Ministry of Health and the Ministry of the Environment recently concluded that BPA was a toxic chemical (Environment Canada 2008).

Problems inherent with reliance on GLP as the standard for choosing data are compounded by the process used by federal agencies to determine membership on science advisory panels. Leading experts qualified by specific experience on the chemical or end points under consideration are often specifically excluded from membership. For example, the U.S. FDA's BPA review panel was identified as an expert panel, when in fact the panel was composed largely of scientists lacking any experience in research with BPA. This process, which appears to consider almost any scientist knowledgeable about a chemical to create bias, makes it vastly more difficult for the panel to integrate scientific data from the relevant literature, especially since, as with BPA, there are almost 1,000 relevant studies and the review panel is provided with very little time to become knowledgeable about the details. It means that the depth of knowledge present on this and similarly constituted government regulatory agency panels is unlikely to be sufficient to subject draft assessments to the scrutiny that peer review by experts normally entails. Combined with reliance on GLP data, this process has a high potential to yield flawed assessments that jeopardize public health.

We are not suggesting that GLP should be abandoned as a requirement for industry-funded studies. We object, however, to regulatory agencies implying that GLP indicates that industry-funded GLP research is somehow superior to NIH-funded studies that are not conducted using GLP. This argument demonstrates a lack of understanding of the profound difference between the use of replication as a mechanism to assess reliability and the methods used to assess validity for peer-reviewed published academic studies, whereas GLP was instituted with the expectation that this type of verification would not occur.

Public health decisions should be based on studies using appropriate protocols and the most sensitive assays. They should not be based on criteria that include or exclude data depending on whether or not the studies use GLP. Simply meeting GLP requirements is insufficient to guarantee scientific reliability and validity.

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IDENTIFICATION OF GENES THAT RESTRICT ASTROCYTE DIFFERENTIATION OF MIDGESTATIONAL NEURAL PRECURSOR CELLS

T. SANOSAKA,[a] M. NAMIHIRA,[a] H. ASANO,[a] J. KOHYAMA,[a] K. AISAKI,[b] K. IGARASHI,[b] J. KANNO[b] AND K. NAKASHIMA[a]*

[a]*Laboratory of Molecular Neuroscience, Graduate School of Biological Sciences, Nara Institute of Science and Technology, 8916-5, Takayama, Ikoma, Nara 630-0101, Japan*

[b]*Division of Cellular and Molecular Toxicology, Biological Safety Research Center, National Institutes of Health Sciences, 1-18-1, Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan*

Abstract—During development of the mammalian CNS, neurons and glial cells (astrocytes and oligodendrocytes) are generated from common neural precursor cells (NPCs). However, neurogenesis precedes gliogenesis, which normally commences at later stages of fetal telencephalic development. Astrocyte differentiation of mouse NPCs at embryonic day (E) 14.5 (relatively late gestation) is induced by activation of the transcription factor signal transducer and activator of transcription (STAT) 3, whereas at E11.5 (mid-gestation) NPCs do not differentiate into astrocytes even when stimulated by STAT3-activating cytokines such as leukemia inhibitory factor (LIF). This can be explained in part by the fact that astrocyte-specific gene promoters are highly methylated in NPCs at E11.5, but other mechanisms are also likely to play a role. We therefore sought to identify genes involved in the inhibition of astrocyte differentiation of NPCs at midgestation. We first examined gene expression profiles in E11.5 and E14.5 NPCs, using Affymetrix GeneChip analysis, applying the Percellome method to normalize gene expression level. We then conducted *in situ* hybridization analysis for selected genes found to be highly expressed in NPCs at midgestation. Among these genes, we found that *N-myc* and high mobility group AT-hook 2 (*Hmga2*) were highly expressed in the E11.5 but not the E14.5 ventricular zone of mouse brain, where NPCs reside. Transduction of *N-myc* and *Hmga2* by retroviruses into E14.5 NPCs, which normally differentiate into astrocytes in response to LIF, resulted in suppression of astrocyte differentiation. However, sustained expression of *N-myc* and *Hmga2* in E11.5 NPCs failed to maintain the hypermethylated status of an astrocyte-specific gene promoter. Taken together, our data suggest that astrocyte differentiation of NPCs is regulated not only by DNA methylation but also by genes whose expression is controlled spatio-temporally during brain development.

*Corresponding author. Tel: +81-743-72-5471; fax: +81-743-72-5479.
E-mail address: kin@bs.naist.jp (K. Nakashima).

Abbreviations: bHLH, basic helix–loop–helix; BMP, bone morphogenetic protein; CNTF, ciliary neurotrophic factor; CT-1, cardiotrophin-1; DIG, digoxigenin; E, embryonic day; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; GEO, Gene Expression Omnibus; *gfap*, glial fibrillary acidic protein; *Hmga2*, high mobility group AT-hook 2; JAK, janus kinase; LIF, leukemia inhibitory factor; NPC, neural precursor cell; SSC, sodium chloride sodium citrate; STAT, signal transducer and activator of transcription.

Key words: *N-myc*, *Hmga2*, epigenetics, Percellome method, differentiation.

The mammalian CNS is composed of neurons, astrocytes, and oligodendrocytes. Although these three cell types are derived from common multipotent neural precursor cells (NPCs), their differentiation is spatially and temporally regulated during development (Temple, 2001). Fetal telencephalic NPCs divide symmetrically in early gestation to increase their own numbers, and then undergo neurogenesis through mostly asymmetric divisions. Toward the end of the neurogenic phase, NPCs acquire multipotentiality to generate astrocytes and oligodendrocytes as well as neurons. It has recently become apparent that NPC fate determination is controlled by both extracellular cues, including cytokine signaling, and intracellular programs such as epigenetic gene regulation (Edlund and Jessell, 1999; Takizawa et al., 2001; Hsieh and Gage, 2004).

Interleukin (IL) -6 family cytokines such as cardiotrophin-1 (CT-1), leukemia inhibitory factor (LIF) and ciliary neurotrophic factor (CNTF) activate the janus kinase (JAK) –signal transducer and activator of transcription (STAT) signaling pathway and are known to induce astrocyte differentiation of NPCs (Bonni et al., 1997; Rajan and McKay, 1998). Gene knockouts of LIF (Bugga et al., 1998), LIF receptor β (Koblar et al., 1998), the common receptor component gp130 (Nakashima et al., 1999a) and STAT3 (He et al., 2005) all result in impaired astrocyte differentiation *in vivo*, emphasizing the contribution of JAK-STAT signaling to astrogliogenesis in the developing CNS. Bone morphogenetic proteins (BMPs) are another group of astrocyte-inducing cytokines. They synergistically induce astrocytic differentiation of NPCs via formation of a complex between STATs and BMP-activated transcription factor Smads, bridged by the transcriptional coactivators p300/CBP (Nakashima et al., 1999b).

In addition to these extracellular factors, intracellular programs and factors also play critical roles to regulate astrocytic differentiation of NPCs. We have previously shown that a CpG dinucleotide within a STAT3-binding element (TTC*CG*AGAA) in the astrocytic marker glial fibrillary acidic protein (*gfap*) gene promoter is highly methylated in NPCs at midgestation (embryonic day (E)11.5), when the cells differentiate only into neurons but not into astrocytes. Since STAT3 does not bind to the methylated cognate sequence, NPCs at midgestation do not express *gfap* even when stimulated by STAT3-activating cytokines such as LIF. As gestation proceeds, the STAT3-binding

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site becomes gradually demethylated in NPCs, enabling them to express *gfap* in response to LIF stimulation (Takizawa et al., 2001). Thus, we have proposed that DNA methylation is a critical cell-intrinsic determinant of astrocyte differentiation during brain development. However, the important question of how this astrocyte-specific gene promoter becomes demethylated in NPCs remains unanswered.

Neurogenic basic helix–loop–helix (bHLH) transcription factors have been also shown to regulate astrocyte differentiation during early neural development. Mice carrying mutations in *mash1* and *math3* (Tomita et al., 2000), or, to a lesser extent, *mash1* and *ngn2* (Nieto et al., 2001) exhibit decreased neurogenesis and premature astrogliogenesis. Conversely, overexpression of neurogenic bHLH factors, either *in vivo* during the gliogenic period (Cai et al., 2000) or in cultured NPCs exposed to CNTF (Sun et al., 2001), promotes neurogenesis at the expense of astrogliogenesis. A possible mechanism underlying the repressive effect on astrogliogenesis is that Ngn1 binds to p300/CBP and sequesters them away from STAT3, thereby preventing STAT3 from activating astrocytic gene expression (Sun et al., 2001). Such a mechanism may ensure the restriction of astrocyte differentiation in NPCs that would otherwise differentiate into neurons under the influence of high-level neurogenic bHLH factor expression during the neurogenic period.

Although these studies have provided us with an integrated insight into the mechanism of neurogenic-to-gliogenic switching in NPCs, they do not preclude the involvement of other, as yet unknown, factors. To identify such factors, we first in this study examined gene expression profiles of mid- and late-gestational NPCs by Affymetrix GeneChip analysis, which is widely used to obtain a complete picture of developmental stage-specific gene expression (Abramova et al., 2005; Ajioka et al., 2006). We then performed *in situ* hybridization experiments to investigate the spatio-temporal expression pattern of genes that were found to be highly expressed in midgestational NPCs. Two genes, *N-myc* and high mobility group AT-hook 2 (*Hmga2*), were highly expressed in the ventricular zone of E11.5 but not of E14.5 mouse brain. Transduction of *N-myc* and *Hmga2* into E14.5 NPCs resulted in suppression of astrocyte differentiation, even in the presence of LIF. However, the prolonged expression of these genes in E11.5 NPCs failed to preserve the hypermethylated status of the astrocyte-specific *gfap* promoter. These results suggest that the inhibition of astrocyte differentiation in midgestational NPCs is regulated not only by DNA methylation of astrocyte-specific gene promoters but also by transcription-regulating factors whose expression is controlled spatiotemporally during brain development.

EXPERIMENTAL PROCEDURES

NPC culture

Timed-pregnant ICR mice were used to prepare NPCs. The protocols described below were carried out according to the animal experimentation guidelines of Nara Institute of Science and Technology that comply with National Institutes of Health Guide for Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals used and their suffering. NPCs were prepared from telencephalons of E11.5 and E14.5 mice and cultured as described previously (Nakashima et al., 1999b). Briefly, the telencephalons were triturated in Hanks' balanced salt solution by mild pipetting with a 1-ml pipet tip (Gilson, Middleton, WI, USA). Dissociated cells were cultured in N2-supplemented Dulbecco's Modified Eagle's Medium with F12 (GIBCO, Grand Island, NY, USA) containing 10 ng/ml basic FGF (R&D Systems, Minneapolis, MN, USA) (N2/DMEM/F12/bFGF) on culture dishes (Nunc, Naperville, IL, USA) or chamber slides (Nunc) which had been precoated with poly-L-ornithine (Sigma, St. Louis, MO, USA) and fibronectin (Sigma).

Immunocytochemistry

E11.5 and E14.5 NPCs cultured on coated chamber slides were washed with PBS, fixed in 4% paraformaldehyde in PBS, and stained with the following primary antibodies: rabbit anti-SOX2 (1:1000, Chemicon, Temecula, CA, USA), mouse anti-βIII-tubulin (1:500, Sigma), rabbit anti-GFAP (1:2000, Dako, High Wycombe, UK). The following secondary antibodies were used: Alexa488-conjugated goat anti-rabbit IgG (1:500, Molecular Probes, Eugene, OR, USA), Cy3-conjugated goat anti-mouse IgG (1:500, Chemicon). Nuclei were stained using bisbenzimide H33258 fluorochrome trihydrochloride (Nacalai Tesque, Kyoto, Japan). All experiments were independently replicated at least three times.

Sample preparation and GeneChip analysis

These procedures were conducted according to the Percellome method (Kanno et al., 2006) to normalize mRNA expression values to sample cell numbers by adding external spike mRNAs to the sample in proportion to the genomic DNA concentration and utilizing the spike RNA quantity data as a dose-response standard curve for each sample. Cells cultured on coated dishes were washed with PBS, lysed in 500 ml of RLT buffer (Qiagen K.K., Tokyo, Japan) and transferred to a 1.5-ml tube. Two separate 10-μl aliquots were treated with DNAse-free RNase A (Nippon Gene, Tokyo, Japan) for 30 min at 37 °C, followed by proteinase K (Roche Diagnostics, Mannheim, Germany) for 3 h at 55 °C, and then transferred to a 96-well black plate. PicoGreen fluorescent dye (Molecular Probes) was added to each well, and then incubated for 2 min at 30 °C. The DNA concentration was measured using a 96-well fluorescence plate reader with excitation at 485 nm and emission at 538 nm. Lamda phage DNA (PicoGreen kit, Molecular Probes) was used as standard. The appropriate amount of spike RNA cocktail was added to the sample homogenates in proportion to their DNA concentration. Five independent *Bacillus subtilis* poly-A RNAs were included in the grade-dosed spike cocktail. Total RNAs were purified using an RNeasy Mini kit (Qiagen), according to the manufacturer's instructions. First-strand cDNAs were synthesized by incubating 5 μg of total RNA with 200 U SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and 100 pmol T7-$(dT)_{24}$ primer [5′-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG-$(dT)_{24}$-3′]. After second-strand synthesis, the double-stranded cDNAs were purified using a GeneChip Sample Cleanup Module (Affymetrix, Washington, DC, USA), according to the manufacturer's instructions, and labeled by *in vitro* transcription using a BioArray HighYield RNA transcript labeling kit (Enzo Life Sciences, Farmingdale, NY, USA). The labeled cRNA was then purified using a GeneChip Sample Cleanup Module (Affymetrix) and treated with fragmentation buffer at 94 °C for 35 min. For hybridization to a GeneChip Mouse Genome 430 2.0 Array (Affymetrix), 15 μg of fragmented cRNA probe was incubated with 50 pM control oligonucleotide B2, 1× eukaryotic hybridization control (1.5 pM BioB, 5 pM BioC, 25 pM BioD and 100 pM Cre), 0.1 mg/ml herring sperm

DNA, 0.5 mg/ml acetylated BSA and 1× manufacturer-recommended hybridization buffer in a 45 °C rotisserie oven for 16 h. Washing and staining were performed in a GeneChip Fluidics Station (Affymetrix) using the appropriate antibody amplification, washing and staining protocols. The phycoerythrin-stained arrays were scanned as digital image files, which were analyzed with GeneChip Operating Software (Affymetrix). The expression data were converted to copy numbers of mRNA per cell by the Percellome method, quality controlled, and analyzed using Percellome software (Kanno et al., 2006). The GeneChip data have been deposited in the NCBI Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) and is accessible through GEO series accession number GSE 10796.

Quantitative real-time RT-PCR

Quantitative real-time PCR was performed to confirm the results of GeneChip analysis. RNAs from E11.5 and E14.5 NPCs were reverse transcribed using Superscript II (Invitrogen) and amplified by PCR, with a specific pair of primers for each gene, using the Mx3000P system (Stratagene, La Jolla, CA, USA). The expression of target genes was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*). The gene-specific primers were as follows: mouse N-myc: N-myc-S, 5′-aactatgctgcaccctcacc-3′; N-myc-AS, 5′-tagcaagtccgagcgtgttc-3′; mouse Hmga2: Hmga2-S, 5′-ggcagccgtccacatcag-3′; Hmga2-AS, 5′-taatcctcctctgcggactc-3′; mouse Sox11: Sox11-S, 5′-gagcctgtacgacgaagtgc-3′; Sox11-AS, 5′-tgaacaccaggtcggagaag-3′; mouse Bhlhb5: Bhlhb5-S, 5′-gttgcgcctcaacatcaac-3′; Bhlhb5-AS, 5′-acttttgcagaggctggac-3′; mouse Bcl11a: Bcl11a-S, 5′-gcatcaagctggagaaggag-3′; Bcl11a-AS, 5′-gagcttccatccgaaaactg-3′; mouse Gapdh: Gapdh-S, 5′-accacagtccatgccatcac-3′; Gapdh-AS, 5′-tccaccaccctgttgctgta-3′.

In situ hybridization

Digoxigenin- (DIG; Roche) labeled cRNA probes were synthesized for each gene, following the manufacturer's instructions. Cryosections were washed with PBS and fixed with 4% PFA. After fixation, sections were incubated in prehybridization solution (5× sodium chloride sodium citrate (SSC), 1% SDS, 50 μg/ml yeast transfer RNA, 50 μg/ml heparin in 50% formamide) at 70 °C for 1 h and hybridized with 500 ng/ml of DIG-labeled cRNA probes at 65 °C for 16 h. After three washes with wash solution 1 (5× SSC, 1% SDS in 50% formamide) and wash solution 3 (2× SSC in 50% formamide), sections were blocked with 10% normal sheep serum in TBST at room temperature for 1 h and then incubated with 1:1000 alkaline phosphatase-conjugated anti-DIG antibody (Roche) at 4 °C for 16 h. After four washes with TBST, hybridized probes were visualized with 5-bromo-4-chloro-3 indolylphosphate and nitro blue tetrazolium chloride.

Recombinant retrovirus construction and infection

Human *N-myc* and mouse *Hmga2* cDNAs were cloned into the expression vector pMYs, which contains an internal ribosome entry site followed by the region upstream of the *EGFP* gene (Morita et al., 2000). The Plat-E packaging cell line was transiently transfected with the retrovirus DNA by Trans-IT 293 (Mirus, Madison, WI, USA) (Morita et al., 2000). On the following day, the medium was replaced with N2/DMEM/F12/bFGF, and the cells were cultured in this medium for 1 day before virus was collected.

Fluorescence activated cell sorting

Virus-infected E11.5 NPCs were cultured for 4 days, after which GFP-labeled cells were sorted using a FACS Vantage (Becton Dickinson, Franklin Lakes, NJ, USA) at a flow rate of less than 1500 events/s; gating parameters were set by side and forward scatter to eliminate debris, dead and aggregated cells. After sorting, genomic DNA was extracted and used for bisulfite sequencing.

Bisulfite sequencing

Sodium bisulfite treatment of genomic DNA was performed using a Methylamp DNA Modification kit (Epigentek, Brooklyn, NY, USA), according to the manufacturer's instructions. The region in the *gfap* promoter containing the STAT-binding site of the bisulfite-treated genomic DNA was amplified by PCR using the following primers: GFmS (5′-GGGATTTATTAGGAGAATTTTAGAAGTAG-3′), GFmAS (5′-TCTACCCATACTTAAACTTCTAATATCTAC-3′). The PCR products were cloned into pT7Blue vector (Novagen, Madison, WI, USA) and at least 12 randomly selected clones were sequenced.

RESULTS

Preparation of NPCs from different developmental stages and comparison of their gene expression profiles by GeneChip analysis

E11.5 NPCs do not differentiate into astrocytes, even in the presence of the astrocyte-inducing cytokine LIF, in contrast to 4-day cultured E14.5 NPCs (Takizawa et al., 2001). As a first step toward identifying factors involved in the inhibition of astrocyte differentiation of NPCs at midgestation, we examined the gene expression profiles of E11.5 and E14.5 NPCs.

E11.5 and E14.5 NPCs were isolated from embryonic telencephalon and cultured as indicated in Fig. 1A. To evaluate the purity of NPCs in each cell population, the cells were stained with antibody against SOX2, an NPC marker (Graham et al., 2003). As shown in Fig. 1B and C, the majority of cells in both populations were positive for SOX2, indicating that NPCs were highly enriched. An Affymetrix mouse genome GeneChip array was chosen to compare expression profiles in the two populations, and we adopted the Percellome method to normalize gene expression from different samples (Kanno et al., 2006). The method enabled us to quantify mRNA molecules per cell based on the measurement of cell by adding a grade-dosed spike cocktail to the samples. We excluded genes whose transcript copy number was below six per cell. Scatter plots illustrating the differences between E11.5 and E14.5 NPCs are shown in Fig. 1D; 194 genes were expressed at >fivefold higher level in E11.5 NPCs than in E14.5 NPCs (Fig. 1D, light blue zone). Of these, 102 were known genes, and were classified by functional category (Fig. 1E). Since we wished to identify negative regulators of astrocyte differentiation, or factors involved in the epigenetic modification in midgestational NPCs, we focused on transcription-related genes (Fig. 1E, red). These 21 genes are listed in Table 1, and five (*N-myc*, *Hmga2*, *Bhlhb5*, *Sox11*, *Bcl11a*) were selected for further analysis because they have been reported to play roles in cell growth, differentiation, and chromatin remodeling in other types of stem cells (Sawai et al., 1990; Zhou et al., 1995; Saiki et al., 2000; Knoepfler et al., 2002; Brunelli et al., 2003; Sock et al., 2004).

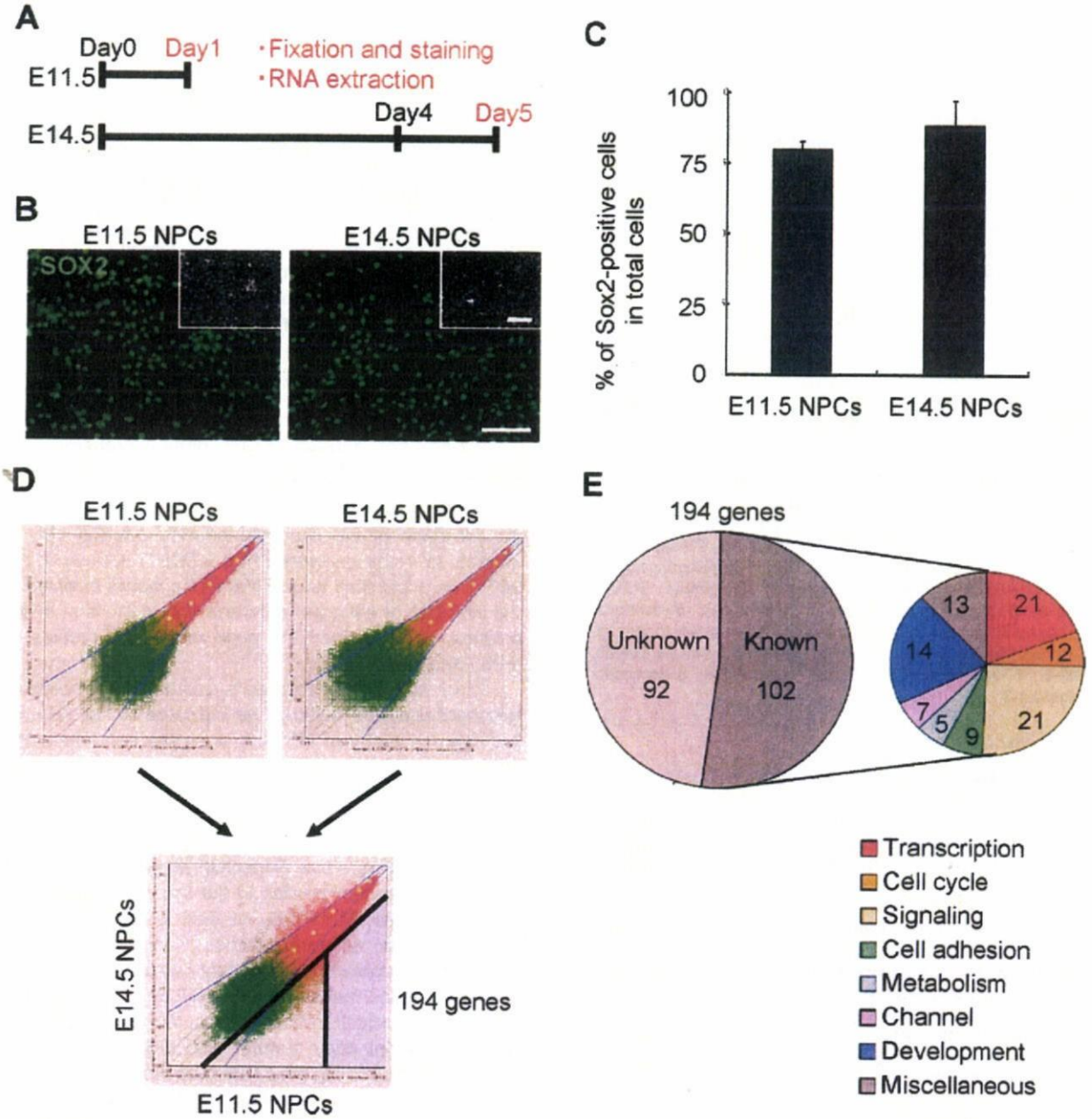

Fig. 1. Comparison of gene expression profiles in E11.5 and E14.5 NPCs. (A) Schematic of experimental protocol. NPCs isolated from E11.5 mouse telencephalon were plated (day 0) and used on the following day for immunostaining and RNA extraction (day 1). NPCs isolated at E14.5 were expanded for 4 days and replated on day 4. On day 5, these cells were fixed for immunostaining. RNA was also extracted. (B) E11.5 and E14.5 NPCs were stained with antibody against Sox2 (green). Scale bar=25 μm. Insets: Hoechst nuclear staining of each field. Scale bar=25 μm. (C) The percentage of Sox2-positive cells in E11.5 and E14.5 NPCs was quantified. Mean±S.D. (D) Scatter plots of E11.5 (upper left) and E14.5 (upper right) samples obtained from GeneChip analysis indicated no significant change between independent experiments with the same sample. Overview (lower plot) of gene expression change was compared between each sample. One hundred ninety-four genes were expressed at >fivefold higher level in E11.5 NPCs than E14.5 NPCs (light blue zone). (E) Of the 194 genes that were highly expressed in E11.5 NPCs, known genes were classified according to Affymetrix gene ontology. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

Table 1. Transcription-related genes highly expressed in E11.5 NPCs

Probe set ID	GenBank ID	Gene symbol	E11.5 NPCs	E14.5 NPCs	E11.5/ E14.5
1433919_at	AV302111	*Asb4*	9.8	0.5	19.6
1419406_a_at	NM_016707	***Bcl11a***	13.8	1.8	7.7
1418271_at	NM_021560	***Bhlhb5***	10.6	1.2	8.8
1452207_at	Y15163	*Cited2*	16.7	2.6	6.4
1449470_at	NM_010053	*Dlx1*	13.8	2.4	5.8
1448877_at	NM_010054	*Dlx2*	9.8	1.8	5.4
1449863_a_at	NM_010056	*Dlx5*	11.2	0.7	16.0
1459211_at	AW546128	*Gli2*	8.0	1.5	5.3
1456067_at	AW546010	*Gli3*	20.6	2.1	9.8
1422851_at	X58380	***Hmga2***	25.5	0.5	51.0
1450723_at	BQ176915	*Ils1*	8.6	0.1	86.0
1427300_at	D49658	*Lhx8*	10.5	0.1	105.0
1417155_at	BC005453	***N-myc***	8.2	1.2	6.8
1415811_at	BB702754	*NP95*	12.6	2.1	6.0
1421193_a_at	NM_016768	*Pbx3*	12.3	1.6	7.7
1417400_at	NM_030690	*Rai14*	11.0	1.6	6.9
1435856_x_at	AV310148	*Smarcb1*	8.0	1.6	5.0
1431255_at	BB656631	***Sox11***	38.7	6.2	6.2
1450034_at	AW214029	*Stat1*	9.6	1.8	5.3
1416711_at	NM_009322	*Tbr1*	9.8	0.2	49.0
1423424_at	BB732077	*Zic3*	11.2	0.5	22.4

Genes reported to participate in cell growth, differentiation and chromatin remodeling are shown in boldface.

Spatio-temporal expression patterns of genes highly expressed in E11.5 NPCs

To substantiate the GeneChip results, we extracted RNA from E11.5 and E14.5 NPCs and performed real-time RT-PCR using specific primers for each selected gene. Consistent with the GeneChip analysis, all five genes were highly expressed in E11.5 NPCs compared with E14.5 NPCs (Fig. 2A). We next performed *in situ* hybridization for each gene using E11.5, E14.5 and E17.5 mouse brain sections (Fig. 2B). *N-myc* and *Hmga2* mRNAs were specifically detected in the ventricular zone (VZ) of E11.5 brain, implying that N-myc and Hmga2 play some role in NPCs at this stage. By contrast, *Bhlhb5*, *Sox11* and *Bcl11a* expression was stronger in cortical plate, where postmitotic neurons reside, than in the VZ (Fig. 2B). We therefore decided to analyze the function of N-myc and Hmga2 in more detail.

Transduction of *N-myc* and *Hmga2* inhibits astrocyte differentiation of E14.5 NPCs

We next examined whether N-myc and Hmga2 affect astrocyte differentiation of NPCs. We expressed EGFP alone (control), and EGFP together with either N-myc or Hmga2, using retroviral infection in E14.5 NPCs, in which expression of the endogenous genes is very low. Virus-infected E14.5 NPCs were cultured for 4 days in the presence of LIF to induce astrocyte differentiation, and then stained with antibodies against GFP and GFAP. As shown in Fig. 3A and B, NPCs infected with control virus effectively differentiated into GFAP-positive astrocytes in response to LIF stimulation (42±2.6%). In contrast, GFAP-positive astrocyte differentiation was virtually abolished in cells ectopically expressing N-myc (0.5±0.4%) and Hmga2 (3±2.0%) (Fig. 3A, B). Expression of these genes did not significantly affect neuronal differentiation of NPCs, as assessed by monitoring expression of the neuronal marker βIII-tubulin, compared with the control cells (Fig. 3C, D). We further examined whether the observed suppression of astrocyte differentiation of NPCs infected with viruses encoding N-myc or Hmga2 could be attributed to specific cell-growth inhibition or to cell death. To address this issue, we performed immune staining for the cycling cell marker Ki67 and the apoptotic marker cleaved caspase 3. Although proliferation of NPCs ectopically expressing N-myc or Hmga2 appeared to be slightly enhanced, expression of either gene caused negligible cell death. These results suggest that N-myc and Hmga2 inhibit astrocyte differentiation of NPCs by a mechanism distinct from that of the neurogenic bHLH factors, which enhance neuronal differentiation (Sun et al., 2001).

Continuous expression of N-myc and Hmga2 in E11.5 NPCs fails to preserve the hypermethylated status of an astrocyte-specific gene promoter

We have previously shown that the *gfap* promoter is highly methylated in E11.5 NPCs, and becomes demethylated as gestation proceeds (Takizawa et al., 2001). This demethylation enables NPCs at later developmental stages, E14.5 or thereafter, to respond to LIF and differentiate into GFAP-positive astrocytes. As shown in the foregoing data, expression levels of *N-myc* and *Hmga2* thus seemed to be reduced concurrently with the developmental stage-dependent demethylation of an astrocyte-specific gene promoter; furthermore, ectopic expression of these genes in E14.5 NPCs inhibited GFAP-positive astrocyte differentiation. We therefore hypothesized that sustained expression of N-myc and Hmga2 in E11.5 NPCs might maintain the hypermethylated status of the *gfap* promoter. To test this, we infected E11.5 NPCs with viruses expressing EGFP alone and EGFP together with either N-myc or Hmga2 and cultured them for 4 days. GFP-positive cells were sorted by FACS and their genomic DNAs were extracted for bisulfite sequencing. As observed in the previous study (Takizawa et al., 2001), the *gfap* promoter including the STAT3 site became demethylated to about 65% in control virus-infected cells after the 4-day culture, and this was also the case for both N-myc- and Hmga2-expressing virus-infected cells (Fig. 3E, F). These results indicate that sustained expression of N-myc and Hmga2 in E11.5 NPCs does not affect the process of demethylation in this astrocyte-specific gene promoter. On the other hand, when 4-day-cultured control virus-infected E11.5 NPCs were then stimulated with LIF for an additional 4 days, GFAP-positive astrocytes appeared, probably due to demethylation in the promoter, whereas neither *N-myc* nor *Hmga2* virus-infected cells gave rise to astrocytes even in the presence of LIF (data not shown). These results suggest that N-myc and Hmga2 inhibit precocious astrocyte differentiation of midgestational NPCs independent of the DNA methylation status of an astrocyte-specific gene promoter.

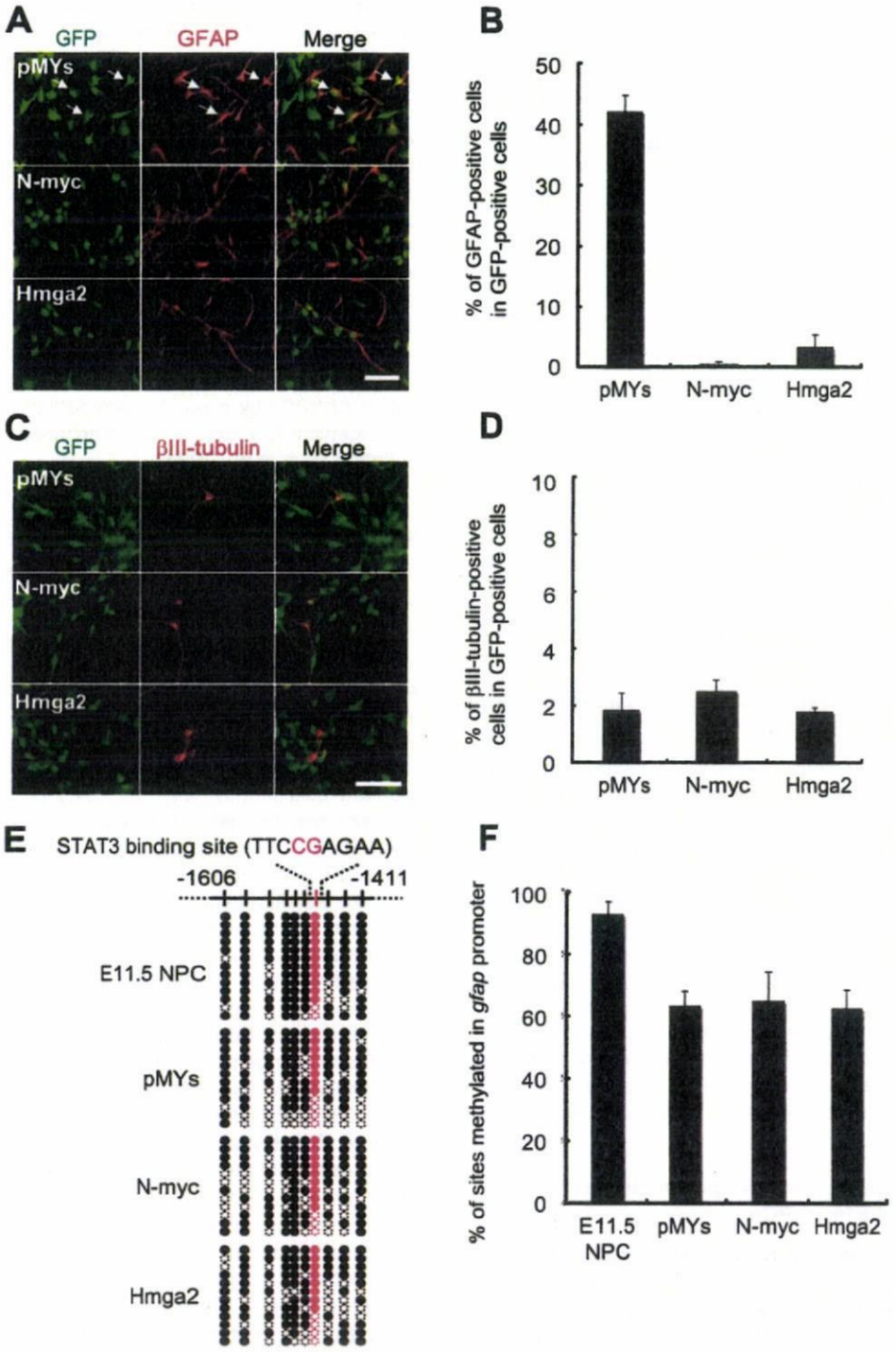

Fig. 3. N-myc and Hmga2 inhibit astrocyte differentiation of NPCs even in the presence of LIF. (A–D) E14.5 NPCs were infected with recombinant retroviruses engineered to express *EGFP* alone (pMYs), and *EGFP* together with either *N-myc* (N-myc) or *Hmga2* (Hmga2), and cultured with LIF (50 ng/ml) for 4 days to induce astrocyte differentiation. The cells were then stained with antibodies against GFP (green) and GFAP (red) (A). Arrows indicate GFP/GFAP double-positive cells. The percentage of GFAP-positive astrocytes in GFP-positive cells was quantified (B). Mean±S.D. The cells were also stained with antibodies against GFP (green) and βIII-tubulin (red) (C). The percentage of βIII-tubulin-positive neurons in GFP-positive cells was quantified (D). Mean±S.D. (E) E11.5 NPCs were infected with recombinant retroviruses engineered to express *EGFP* alone (pMYs), and *EGFP* together with either *N-myc* (N-myc) or *Hmga2* (Hmga2), and subsequently cultured for 4 days. GFP-positive cells were then sorted based on GFP fluorescence, and genomic DNA was extracted for bisulfite sequencing. White and black circles indicate unmethylated and methylated CpG sites, respectively. The E11.5 NPC sample was freshly prepared NPCs from telencephalon at E11.5. The CpG dinucleotide within the STAT3 binding site is indicated in red. (F) Methylation frequency in the *gfap* promoter. Mean±S.D. Scale bars=50 μm (A, C). For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.