に関する検討会においてスクリーニング及びテスティングの2段階からなる「試験スキーム」を提案し、これに適用可能な評価法について、本研究班を始めとした研究班においてこれまで様々な研究開発を進めてきた。その成果の一部は、OECD ガイドライン化などにより国際的な提案を行ってきている。スクリーニング段階では、*in silico*、*in vitro*、*in vivo* のスクリーニング試験結果をもとに化学物質の確定試験実施のための優先順位付けを行うこととなっており、各スクリーニング試験法の妥当性や優先順位付けにおける利用法について検討を行うため、開発がほぼ終了し実施可能な系を用いた大規模スクリーニングを実施している。

4、我が国で実施された大規模スクリーニング試験結果

これまでに我が国では、*in silico* スクリーニング評価法としてエストロゲン及びアンドロゲン受容体のリガンド結合部位の立体構造をもとにした 3 次元ドッキングモデルを用いた、結合性予測法を開発し *in silico* スクリーニングにより、エストロゲン受容体については、約 20,000 構造について、アンドロゲン受容体については、約 4,500 構造について結合予測値を得ている。レポーターアッセイ系については、これまで ERα、ERβ、AR、TR それぞれについてアゴニスト、アンタゴニスト測定系を構築して、それぞれ 750、400、300、250 化合物についての活性測定を実施した。

委託研究

1. ER レポーター遺伝子測定 —ER 系へ作用する化学物質検出法の検証データの収集—（委託先；財団法人 化学物質評価研究機構 安全性評価技術研究所）(H20)

hERα アゴニスト活性測定

実施した 50 化合物のうち、PC10 が算出されたものは 11 物質であり、さらに PC50 も算出されたものは 3 物質であった。

hERα アンタゴニスト活性測定

実施した 50 化合物のうち、IC30 が算出されたものは 7 物質であり、さらに IC50 も算出されたものは 1 物質であった。なお、IC30 が算出された物質について、今回の試験濃度域でいずれも細胞毒性は認められなかった。

2. AhR、AR、TR 系レポーター遺伝子測定 — AR を介する作用に関する研究 —
（委託先；大塚製薬株式会社）**(H20)**

AR アゴニスト活性

これまでの検討から、PC10 が算出できるサンプルは AR アゴニストとして有意な転写活性があると考えられる。よって今回の検討においても、PC10 が算出できる物質をARアゴニストとして定義することとした。測定対象とした 70 物質において、PC10、及び PC20 を算出することができた。prednisolone（ED0812）（PC20 = 1.25×10^{-7}）、piperine（ED0819）（PC10 = 6.74×10^{-6}）、4-amino- azobenzene（ED0855）（PC10 = 6.61×10^{-7}）、resveratrol（ED0868）（PC20 = 6.21×10^{-6}）、mifepristone（RU486）（ED0869）（PC20 = 2.59×10^{-8}）、及び dibenzoylmethane（ED0870）（PC20 = 1.27×10^{-6}）がアゴニストとして検出された。

AR アンタゴニスト活性

AR アッセイのアンタゴニスト活性検出については、これまでの検討により、IC20 が算出できて、なおかつ細胞毒性が 15%未満であれば有意なアンタゴニスト活性が検出されたと判断できる。本年度測定対象とした 70 物質において、有意な AR アンタゴニスト活性

物質は 9 物質であった。 chlornitrofen（ED0864）、bicalutamide （ED0865）、mifepristone （RU486）（ED0869）は比較的強いアンタゴニスト活性を示した。

AR hyper-induction 活性

AR アンタゴニストアッセイ時に、ルシフェラーゼ転写活性が標準物質である DHT の最大レベルを大きく超える化合物が認められた。これら化合物単独での転写活性の上昇はわずかであった。

AR、GR アゴニスト活性（一過性発現系）

AR アゴニスト活性を示した 6 化合物について、GR に対する作用を検討するために、AR 及び GR を CHO-K1 細胞に一過的に発現させてアゴニスト活性を測定した。

AR アゴニスト様活性を示した化合物の中で、prednisolone（ED0812）は、GR に対するアゴニスト活性を示した。また、AR 発現細胞とコントロール細胞で有意な差が見られないことから、prednisolone は GR アゴニストであることが示唆された。また、mifepristone（RU486）（ED0869）は、AR アゴニストとしての作用を示すが、GR アゴニスト作用も同時に示した。

弱い AR アゴニスト活性を示した piperine（ED0819）（PC10 = 6.74×10^{-6}）、4-aminoazobenzene（ED0855）（PC10 = 6.61×10^{-7}）、resveratrol（ED0868）（PC20 = 6.21×10^{-6}）、及び dibenzoylmethane（ED0870）（PC20 = 1.27×10^{-6}）については、一過性発現系においては、アゴニスト様作用を示さなかった。

3. ホルモン活性予測計算 —高感受性集団へ影響を及ぼす化学物質の電算検索—

（委託先；株式会社 医薬分子設計研究所）

（H20）

AR 結合性予測システムを利用した、約 3,000 化合物の予測計算

AR への結合強度の予測計算を実施する化学物質として、既存化学物質を中心に約 3,000 化合物がリストアップされた。ただし、このうち以下のような化合物は計算の対象外とした。

- CAS 番号から化学構造式が得られなかった物質
- 複数の物質の混合物
- 複数の位置異性体を含む物質
- ポリマー
- 金属錯体
- ケイ素、ホウ素、金属など、分子力場パラメタが未整備な元素を含む物質
- 非常にフレキシブルな物質（回転可能な結合が 30 本以上）
- 自動ドッキングの足がかりに利用できる水素結合可能な原子（酸素、窒素）や環構造等を全く含まない物質

AR 結合強度予測計算は 1t63（DHT 結合）、2pnu（EM5744）の 2 つの結晶構造を用いて実施した。まず 1t63 を用いて結合性を予測し、1t63 に対してドッキング不可であった化合物についてのみ、2pnu を用いた予測計算を実施する2段階法により予測計算を行った。AR 予測モデルにおいて logRBA 推算のために用いた式を以下に示す。

1t63:

logRBA（AR-1t63） = −2.213 GBelc − 0.418 GBrep − 0.021 GBcnf − 0.106 Dlig − 0.203 Desolv + 0.125 GBsole + 0.091 GBsolb − 5.523 （1）

2pnu:

logRBA（AR-2pnu） = −2.381 GBelc −

0.479 GBrep − 0.238 GBcnf − 0.122 Dlig − 0.133 Desolv + 0.066 GBsole− 5.880 (2)

GBelc 、GBrep 、GBcnf 、Dlig, Desolv, GBsole、GBsolb:結合自由エネルギー計算で算出される各エネルギー項

GBelc : GenB で計算される分子間静電相互作用エネルギー

GBrep : GenB で計算される分子間立体相互作用エネルギー

GBcnf : GenB で計算されるリガンドの結合に伴う回転結合自由度の束縛効果

Dlig: Bluto で計算されるリガンド分子内エネルギー変化(単独存在時と蛋白結合時とのエネルギー差)

Desolv : Desolv で計算されるリガンド、蛋白双方の複合体形成に伴う水素結合変化

GBsole: GenB で計算されるリガンド脱溶媒和エネルギー

GBsolb: GenB で計算される蛋白脱溶媒和エネルギー

結果として、1t63 構造において 2120 化合物の予測結合値の計算に成功した。さらに 1t63 構造で安定構造が計算されなかった化合物のうち 12 化合物について 2pnu 構造において結合予測値の計算に成功した。

4. 高感受性集団へ影響を及ぼす化学物質の電算検索(委託先;株式会社 医薬分子設計研究所)(H21)

核内受容体結合活性を有する化合物の高速スクリーニング手法として、自動ドッキング法 ADAM を核とした *in silico* スクリーニングにより標的核内受容体の三次元構造情報に基づく、化学物質の結合様式の推定ならびに結合性予測を行った。

平成 21 年度は、国立医薬品食品衛生研究所より供与された化合物リストに従い約 1,500 化学物質について予測計算を実施した。ER 結合モデルでは 1,059 化合物についての予測結合値を得ることが出来た。また、AR 結合モデルでは、1 段階目の 1t63 構造において 953 化合物について、さらに 1t63 構造で安定構造が計算されなかった化合物のうち 45 化合物について 2pnu 構造において結合予測値の計算に成功した。

D. 考察

本研究の特色は、先行実施された「厚生労働省内分泌かく乱化学物質試験スキーム」の策定、受容体原性毒性に基づくと理解される低用量影響の確認、「確定試験」としての「齧歯類一生涯試験法」の開発、受容体原性毒性メカニズム研究、催奇形性メカニズム研究の成果と方法の一部を継承し、また、平成 17 年度より実施された「化学物質による子どもへの健康影響に関する研究－恒常性維持機構発達の過渡特性に立脚したリスク評価研究－(H17-化学-一般-001)」の成果を最大限に利用し、当記目的達成のために、特に受容体原生毒性に関わる分子生物学的検討を通しての研究を実施し、統合する点にある。

本研究班は、先行研究班の構成の一部を継承しつつ、【総括、総合評価スキーム開発研究及び低用量影響研究】を取り纏め部門として、以下、【恒常性維持メカニズムの揺らぎに着目した新評価手法開発研究】、【有害性発現分子メカニズムの解明研究】、【小児など高感受性集団の評価に関する国際動向調査研究】の 3 部門を置き研究を開始した。

【恒常性維持メカニズムの揺らぎに着目した

新評価手法開発研究】では、神経系・免疫系・内分泌系の高次調節率の変動による化学物質の有害性評を評価するための手法を確立する。

神経・行動試験系については、神経障害性に関して必ずしも明確な器質的障害は誘導されないことが想定されたため、本研究班では、高次行動異常を当面の焦点として、齧歯類胎生期・新生児期暴露をモデルとして、記憶保持能力の責任部位である海馬領域に着目した次世代の中枢神経系に及ぼす影響とエピジェネティクス制御の関係を含め多角的に検討したこと、認知機能、場面適応性や報酬効果に及ぼす影響を検査するオペラント条件付けによる行動試験の導入を進めた。免疫系に関しては、有害性指標として自己免疫疾患(人に於いて性差が著しいことで知られるシェーグレン病のモデル)モデルマウスを用いての化学物質暴露による自己免疫発症の有無、及びマウス経胎盤・経母乳 BPA 投与による出生児の免疫系への影響をLocal Lymph Node Assayを用いて検討し、影響評価法としての有用性が得られた。

内分泌系に関しては、周産期化学物質暴露の影響を従前の生殖毒性に限定せず、生殖関連臓器の形成、発達、機能、その加齢変化に対する影響、及び成熟後の遅発性影響の解析を行った。

本研究により、一生涯(発生、発達、成熟、老化)の全ての段階に於ける内分泌かく乱作用を考慮する必要が示されたと同時に、この方向性に沿って引き続き網羅的な確認を加えつつ研究を進めることで、クロストーク問題、低用量問題等に的確に対応可能な確定試験を確立することが出来る見通しが立ったと考える。

【有害性発現分子メカニズムの解明研究】では、恒常性維持機構に対する影響の発現機序を分子・遺伝子レベルで解明し、評価試験の基礎を固める。

胎生期初期影響のメカニズム情報提供支援体制としての胚性幹細胞(ES 細胞)の *in vitro* での多分化能に対する影響解析研究としてES細胞及び胚様体のBPA影響遺伝子を同定、マウスTCDD及びDMSO投与による肝臓遺伝子の網羅的解析、マウス新生児期前立腺のアンドロゲン応答性とエストロゲン受容体の交絡解析、マウス神経幹細胞の成熟に関与する DNA メチル化制御機構を網羅的に解析すること、AhR の抗炎症作用と大腸発がん抑制作用の分子メカニズムを解析すること、化学物質による免疫系活性化が薬物受容体の転写活性化能と体内薬物動態に及ぼす影響について確認した。

【小児など高感受性集団の評価に関する国際動向調査研究】では、OECD 及び WHO 等の国際機関に於ける動向と同調するため、OECDとWHOで進められている基礎研究情報収集及び化学物質暴露における高感受性集団に対応した応有害性評価手法研究の国際動向調査を行うことより評価スキームの確立に資する。

本研究により最先端の分子生命科学の成果と、新評価法開発の成果から高感受性集団に対する有害性評価のための総合的大綱の策定が見込まれる。

少子高齢化が進み、次世代の担い手の確保と、クオリティー・オブ・ライフ(QOL)の向上、社会保障上の負担軽減の一助として、子どもと老人からなる高感受性集団の保護の重要性の認識が今後更に増すものと考えられる。他方、高度先進工業化による新規高性能マテリアルの開発と実用化は、多種少量生産化合物による国民の暴露の機会を増加させると考えられる。暴露事故の未然防

止は産業経済活動の健全成長に大きく貢献するものであるが、特に低用量問題を含む高感受性集団への配慮は厚生労働のみならず経済の重要課題となる。本研究の目標とする高感受性有害性総合評価大綱の策定は、暴露を受ける側と暴露する物質の側の両者の多様化という近未来特性に照らし、国民の安全性確保及び経済活動の健全な発展に大きく貢献すると期待される。

E. 結論

最先端分子生命科学研究及び評価法開発の成果から、高感受性有害性総合的大綱の策定の方向性と基盤が形成され、本研究の継続が今後の国民の安全性確保及び経済活動の健全な発展に貢献するものと期待される。

F. 研究発表

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G. 知的所有権の取得状況

1. 特許取得

Non-RI LLNA 法の感度上昇法
出願番号 : 特許出願 2004-230151
公開番号 : 特許公開 2006-42702

2. 実用新案登録

無し

3. その他

無し

別添 4

Ⅱ．研究成果の刊行に関する一覧表

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III. 研究成果の刊行物・別刷

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Dietary exposure to low doses of bisphenol A: effects on reproduction and development in two generations of C57BL/6J mice.

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Abstract

ABSTRACT This study was conducted to examine the effects of low-dose exposure to bisphenol A on reproduction and development in two generations of mice. Pregnant female C57BL/6J mice (F(0)) were fed a diet containing low doses of bisphenol A (0, 0.33, 3.3, or 33 ppm) from gestational day 6 through postnatal day 22, and the weanlings (F(1) and F(2)) from each F(0) and F(1) dam group, respectively, were also fed these same concentrations of bisphenol A ad libitum until sacrifice. There were no treatment-related changes in body weight, body weight gain, food consumption, gestation length, or the number of live births on postnatal day 1 in F(0) dams between the control group and bisphenol A groups. Sex ratio and viability were similar in all F(1) pups. No treatment-related changes were observed in body weight, food consumption, developmental parameters, anogenital distance, or weight of any of the organs (liver, kidney, heart, spleen, thymus, testis, ovary, or uterus) in F(1) and F(2) adults in either sex. The epididymis weight was slightly higher with 0.33 and 3.3 ppm in F(1) males, but this slight increase was neither dose dependent nor seen across generations. There were no treatment-related effects of bisphenol A on cauda epididymal sperm count or sperm motility in F(1) or F(2) males. These findings indicate that dietary exposure to bisphenol A between 0.33 and 33 ppm does not adversely affect reproduction or development as assessed in two generations of mice.

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FULL PAPER *Pathology*

Gene Expression Profiling and Cellular Distribution of Molecules with Altered Expression in the Hippocampal CA1 Region after Developmental Exposure to Anti-Thyroid Agents in Rats

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ABSTRACT. To determine whether developmental hypothyroidism causes permanent disruption of neuronal development, we first performed a global gene expression profiling study targeting hippocampal CA1 neurons in male rats at the end of maternal exposure to anti-thyroid agents on weaning (postnatal day 20). As a result, genes associated with nervous system development, zinc ion binding, apoptosis and cell adhesion were commonly up- or down-regulated. Genes related to calcium ion binding were up-regulated and those for myelination were often down-regulated. We, then, examined immunohistochemical cellular distribution of Ephrin type A receptor 5 (EphA5) and Tachykinin receptor (Tacr)-3, those selected based on the gene expression profiles, in the hippocampal formation at the adult stage (11-week-old) as well as at the end of exposure. At weaning, both EphA5- and Tacr3-immunoreactive cells with strong intensities appeared in the pyramidal cell layer or stratum oriens of the hippocampal CA1 region. Although the magnitude of the change was decreased at the adult stage, Tacr3 in the CA1 region showed a sustained increase in expressing cells until the adult stage after developmental hypothyroidism. On the other hand, EphA5-expressing cells did not show sustained increase at the adult stage. The results suggest that developmental hypothyroidism caused sustained neuronal expression of Tacr3 in the hippocampal CA1 region, probably reflecting a neuroprotective mechanism for mismigration.

KEY WORDS: developmental hypothyroidism, EphA5, hippocampal CA1 region, Tacr3.

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Thyroid hormones are essential for normal fetal and neonatal brain development. They control neuronal and glial proliferation in definitive brain regions and regulate neural migration and differentiation [12, 18, 21]. In humans, maternal hypothyroxinemia, early in pregnancy, may have adverse effects on fetal brain development and importantly, even mild-moderate hypothyroxinemia may result in suboptimal neurodevelopment [4]. These results may increase the concern of thyroid hormone-disrupting chemicals in the environment.

Experimentally, developmental hypothyroidism leads to growth retardation, neurological defects and impaired performance on a variety of behavioral learning actions [1, 2]. Rat offspring exposed maternally to anti-thyroid agents such as 6-propyl-2-thiouracil (PTU) show brain retardation, with impaired neuronal migration and white matter hypoplasia involving limited axonal myelination and oligodendrocytic accumulation [6, 8, 21]. The outcome of this type of brain retardation is permanent and is accompanied by apparent structural and functional abnormalities. However, it is still unclear whether the molecular aberrations remain in the retarded brain after maturation.

Histological lesion-specific gene expression profiling provides valuable information on the mechanisms underlying lesion development. We have established molecular analysis methods for DNA, RNA and proteins in paraffin-embedded small tissue specimens utilizing an organic solvent-based fixative, methacarn, with high performance close to that achieved with unfixed frozen tissue specimens [22, 26, 27]. We have previously applied these techniques to analyze global gene expression changes in microdissected lesions [23, 28].

Hippocampal CA1 region is a well-known target of developmental hypothyroidism [8], and we, in our recent study, detected a distribution variability of hippocampal CA1 pyramidal neurons reflecting mismigration in rat offspring at the adult stage after developmental exposure to anti-thyroid agents [24]. The present study was performed to determine whether developmental hypothyroidism triggers sustained aberrations in neuronal development associated with neuronal mismigration until the adult stage. For this purpose, we first performed a global gene expression profiling of the CA1-pyramidal cell layer in rat offspring at the end of developmental exposure to anti-thyroid agents. To distinguish chemical-specific expression changes from hypothyroidism-linked ones, two different anti-thyroid

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agents, PTU and 2-mercapto-1-methylimidazole (MMI), were used, and dose-related responses were also examined with PTU. To extract the neuronal cell layer-specific gene expression profile, microdissection technique was applied for microarray analysis. Based on the expression profiles obtained, cellular localization of the molecules showing altered expression were then immunohistochemically examined in the hippocampus at the adult stage as well as at the end of the developmental exposure.

MATERIALS AND METHODS

Chemicals and animals: 6-propyl-2-thiouracil (PTU; CAS No. 51–52–5) and methimazole (2-mercapto-1-methylimidazole: MMI; CAS No. 60–56–0) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Pregnant Crj:CD®(SD)IGS rats were purchased from Charles River Japan Inc. (Yokohama, Japan) at gestation day (GD) 3 (appearance of vaginal plugs was designated as GD 0). Animals were housed individually in polycarbonate cages with wood chip bedding, maintained in an air-conditioned animal room (temperature: 24 ± 1°C; relative humidity: 55 ± 5%) with a 12-hr light/dark cycle and allowed *ad libitum* access to food and tap water. A soy-free diet (Oriental Yeast Co., Ltd., Tokyo, Japan) was chosen as the basal diet for the maternal animals to eliminate possible phytoestrogen effects [10], and water was given *ad libitum* throughout the experimental period including the 1-week acclimation period.

Animal experiments: The animal experiments were identical to those in a previous study [24]. In brief, maternal animals were randomly divided into four groups including untreated controls. Eight dams per group were treated with 3 or 12 ppm of PTU or 200 ppm of MMI in the drinking water from GD 10 to postnatal day (PND) 20 (PND 0: the day of delivery). On PND 2, the litters were culled randomly, leaving four male and four female offspring. On PND 20, 20 male and 20 female offspring (at least one male and one female per dam) per group were subjected to prepubertal necropsy [13, 24].

The remaining animals were maintained until postnatal week (PNW) 11. All offspring consumed the CRF-1 basal diet and tap water *ad libitum* from PND 21 onwards. At PNW 11, all pups were subjected to adult stage necropsy [13, 24].

All animals used in the present study were weighed and sacrificed by exsanguination from the abdominal aorta under deep anesthesia. These protocols were reviewed in terms of animal welfare and approved by the Animal Care and Use Committee of the National Institute of Health Sciences, Japan.

Preparation of tissue specimens and microdissection: For microarray and subsequent real-time RT-PCR analyses, the whole brain of male offspring was removed at prepubertal necropsy on PND 20 (n=4/group) and was fixed with methacarn solution for 2 hr at 4°C [22]. Coronal brain slices taken at the position of –3.5 mm from the bregma were dehydrated and embedded in paraffin. The embedded tissue blocks were stored at 4°C until tissue sectioning for microdissection [9].

For microdissection, 4-μm-thick sections between ten 20 μm-thick serial sections were prepared. The 4 μm-thick sections were stained with hematoxylin and eosin for confirmation of anatomical orientation of the hippocampal substructure to aid microdissection. The 20 μm-thick sections were mounted onto PEN-foil film (Leica Microsystems GmbH, Welzlar, Germany) overlaid on glass slides, dried in an incubator overnight at 37°C, and then stained using an LCM staining kit (Ambion, Inc., Austin, TX, U.S.A.). Bilateral sides of the hippocampal CA1 pyramidal cell layer in the sections were subjected to laser microbeam microdissection (Leica Microsystems GmbH) (Fig. 1). Twenty sections from each animal were used for microdissection, and the bilateral microdissected samples were collected and stored in separate 1.5-m*l* sample tubes at –80°C until the extraction of total RNA.

RNA preparation, amplification and microarray analysis: Total RNA extraction from hippocampal CA1 samples, quantitation of the RNA yield, and amplification of RNA samples were performed using previously described methods [9, 28].

For microarray analysis, second-round-amplified biotin-labeled antisense RNAs were subjected to hybridization with a GeneChip® Rat Genome 230 2.0 Array (Affymetrix, Inc., Santa Clara, CA, U.S.A.), as previously described [28].

The selection of genes and normalization of the expression data were performed using GeneSpring® software (ver7.2, Silicon Genetics, Redwood City, CA, U.S.A.). Per chip normalization was performed according to a previously described method [28]. Genes showing signals judged to be "absent" in all eight samples of untreated controls and in the anti-thyroid agent-exposed group were excluded. Genes

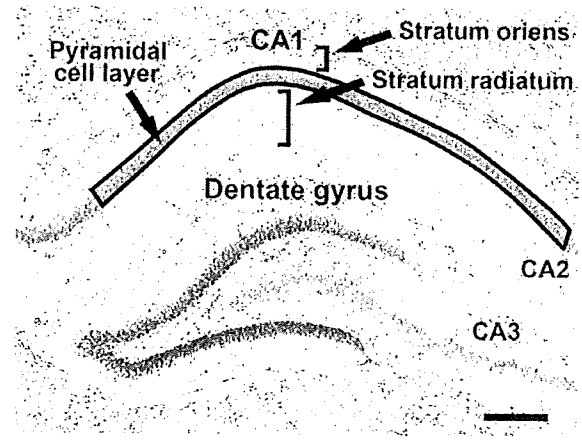

Fig. 1. Overview of the hippocampal formation of a male rat at postnatal day 20 stained with hematoxylin and eosin. Bar=200 μm. The CA1 pyramidal cell layer, enclosed by a solid line, was microdissected for the microarray and subsequent real-time RT-PCR analyses. The number of cells immunoreactive for the candidate molecules in this area was normalized for the length of CA1 used.

showing expression changes with differences of at least twofold in magnitude from the untreated controls were selected, and the "presence" signal in more than 3/4 of samples in each group showing higher expression values were selected. Genes showing altered expression in common in the anti-thyroid agent-exposed groups were also selected.

Real-time RT-PCR: Quantitative real-time RT-PCR was performed to confirm the expression values obtained with microarrays using an ABI Prism 7000 Sequence Detection System (Applied Biosystems Japan, Tokyo, Japan). Genes those showing altered expression (≥2-fold, ≤0.5-fold) in common in the anti-thyroid agent-exposed groups as compared with untreated control offspring were randomly selected, irrespective of the presence or absence of statistically significant difference. As a result, the following seven genes (four up-regulated and three down-regulated) with known function were selected as targets: Tachykinin receptor 3 (*Tacr3*), Calbindin 1, Slit homolog 2 (Drosophila) and Pleomorphic adenoma gene-like 1 (*Plagl1*) as up-regulated examples, and Myelin-associated oligodendrocytic basic protein (*Mobp*), Endothelial differentiation, sphingolipid G-protein-coupled receptor, 8 and CCAAT/enhancer binding protein as down-regulated. RT was performed using first-round antisense RNAs prepared for microarray analysis. For real-time PCR analysis of the genes selected, ABI Assays-on-Demand™ TaqMan® probe and primer sets from Applied Biosystems (available at https://products.appliedbiosystems.com/ab/en/US/adirect/ab?cmd=catNavigate2&catID=601267)(n=4/group) were used. For quantification of the expression data, a standard curve method was applied. The expression values were normalized to two housekeeping genes, Glyceraldehyde 3-phosphate dehydrogenase and Hypoxanthine-guanine phosphoribosyltransferase.

Immunohistochemistry: To evaluate the immunohistochemical distribution of the molecules selected by microarray analysis, the brains of male pups obtained at PND 20 or PNW 11 were fixed in Bouin's solution at room temperature overnight. Six animals were used as untreated controls, six for 200 ppm MMI, eight for 3 ppm PTU, and nine for 12 ppm PTU on PND 20. On PNW 11, 10 animals were used as untreated controls and 10 for 200 ppm MMI, nine for 3 ppm PTU, and six for 12 ppm PTU.

Immunohistochemistry was performed on the brain tissue sections of PND 20 and PNW 11 animals with antibodies against Ephrin type A receptor 5 (EphA5; rabbit IgG, 1:50; Abcam, Cambridge, U.K.) and Tacr3 (rabbit polyclonal antibody, 1:3,000, Novus Biologicals, Inc., Littleton, CO, U.S.A.), which were incubated with the tissue sections overnight at 4°C. Antigen retrieval treatment was not performed for these antigens. Immunodetection was carried out using a VECTASTAIN® Elite ABC kit (Vector Laboratories Inc., Burlingame, CA, U.S.A.) with 3,3'-diaminobenzidine/H_2O_2 as the chromogen, as previously described [23]. The sections were then counterstained with hematoxylin and coverslipped for microscopic examination.

With regard to EphA5, *Efna5*, a gene encoding the representative ligand for this receptor molecule [5], was found to be up-regulated (≥2-fold) by microarray analysis in all of the groups exposed to anti-thyroid agents in the present study (Table 1). Because distribution of EphA5 has been confirmed in the pyramidal cells of the hippocampal CA1 region at both developmental and adult stages in mice and at adult stage in humans [3, 17], we selected this molecule to examine distribution changes in the present study. Tacr3 was also up-regulated in all of the MMI and PTU groups by microarray analysis and real-time RT-PCR in the present study (Table 1). Expression of Tacr3 in the hippocampal CA1 pyramidal neurons has also been confirmed in rats [11], and therefore, we also selected this molecule for examination in the expression changes in the present study.

Morphometry of immunolocalized cells and apoptotic cells: EphA5- or Tacr3-immunoreactive cells distributed in the pyramidal cell layer or stratum oriens of the hippocampal CA1 region were bilaterally counted and normalized to the number in the length of the CA1 region measured (Fig. 1). Tacr3-immunoreactive cells in the subgranular zone of the dentate gyrus were also bilaterally counted and normalized for the number in the length of the granular zone measured. For quantitative measurement of each immunoreactive cellular component, digital photomicrographs at 100-fold magnification were taken using a BX51 microscope (Olympus Optical Co., Ltd., Tokyo, Japan) attached to a DP70 Digital Camera System (Olympus Optical Co., Ltd.), and quantitative measurements were performed using the WinROOF image analysis software package (version 5.7, Mitani Corp., Fukui, Japan).

Statistical analysis: Numerical data of the number of immunoreactive cells were assessed using Student's *t*-test to compare the untreated controls with each of the anti-thyroid agent-exposed groups when the variance was homogenous among the groups using a test for equal variance. If a significant difference in variance was observed, Aspin-Welch's *t*-test was used instead. The data for gene expression levels from real-time RT-PCR analysis were analyzed by the Kruskal-Wallis test, followed by Bartlett's test. When statistically significant differences were indicated, Dunnett's multiple test was used for comparisons with the untreated controls. For the microarray data, statistical analysis was performed with GeneSpring® software, and the significance of gene expression changes was analyzed by Student's *t*-test or ANOVA between the untreated controls and each of the anti-thyroid agent-exposed groups.

RESULTS

Microarray analysis: Figure 2 shows the Venn diagram of genes showing altered expression in the microdissected CA1 pyramidal neurons in the exposure groups in combination or individually in each exposure group. Many genes were found to be up- or down-regulated in common in two of the three groups. The numbers of genes classified into common categories between the groups or individually in each group were similar in terms of up- and down-regulated genes. The number of genes showing up- or down-regula-

Table 1. List of representative genes showing up- or down-regulation common to 2-mercapto-1-methylimidazole (MMI), 3 and 12 ppm 6-propyl-2-thiouracil (PTU) (≥2-fold, ≤0.5-fold)

Gene function	Accession No.	Gene title	Symbol	MMI	3 ppm PTU	12 ppm PTU
Up-regulated genes (of 119 genes in total)						
Nervous system development	AI101660	Slit homolog 2 (Drosophila)	Slit2	3.04	2.62	7.08
Nervous system development	NM_024358.1	Notch gene homolog 2 (Drosophila)	Notch2	2.52	2.01	2.02
Nervous system development	AW527295	Ephrin A5	Efna5	3.12	3.46	4.31
Nervous system development	NM_053465.1	Fucosyltransferase 9	Fut9	2.13	6.75	2.11
Nervous system development	BE106256	Sparc/osteonectin, cwcv and kazal-like domains proteoglycan 1	Spock1	3.22	3.13	2.15
Calcium ion binding	X04280.1	Calbindin 1	Calb1	4.48	4.85	9.00
Calcium ion binding	BM386119	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 3 (GalNAc-T3)	Galnt3	2.43	2.30	2.63
Calcium ion binding	BI279663	Desmocollin 2	Dsc2	2.82	2.04	5.62
Calcium ion binding	AI105369	Calmodulin-like 4	Calml4	3.40	2.25	5.59
Zinc ion binding	BE098686	Similar to Tnf receptor-associated factor 1	LOC687813	3.10	2.04	2.78
Zinc ion binding	BF562032	RAN binding protein 2	Ranbp2	3.49	2.67	2.78
Zinc ion binding	BF397925	ADAMTS-like 1	Adamtsl1	6.22	2.55	7.63
Zinc ion binding	BF395606	Splicing factor, arginine/serine-rich 7	Sfrs7	4.93	2.06	2.90
Apoptosis	NM_012760.1	Pleomorphic adenoma gene-like 1	Plagl1	3.10	4.28	6.86
Apoptosis	NM_057130.1	Harakiri, BCL2 interacting protein (contains only BH3 domain)	Hrk	2.63	2.73	3.18
Cell Adhesion	AA850909	Poliovirus receptor-related 2	Pvrl2	4.74	2.46	2.61
Cell Adhesion	AA819731	Hyaluronan and proteoglycan link protein 4	Hapln4	4.13	6.67	3.46
Cell Adhesion	BI287851	Collagen, type VI, alpha 2	Col6a2	3.45	2.19	5.12
Ion channel activity	AA851939	FXYD domain-containing ion transport regulator 6	Fxyd6	4.73	2.61	7.85
Other	NM_017053.1	Tachykinin receptor 3	Tacr3	7.32	6.19	12.49
Down-regulated genes (of 97 genes in total)						
Nervous system development	NM_031018.1	Activating transcription factor 2	Atf2	0.41	0.36	0.36
Neuron migration	BF390065	Roundabout homolog 3 (Drosophila)	Robo3	0.06	0.31	0.04
Neuron differentiation	AF115249.1	Endothelial differentiation, sphingolipid G-protein-coupled receptor, 8	Edg8	0.40	0.06	0.08
Neuron differentiation	NM_024125.1	CCAAT/enhancer binding protein (C/EBP), beta	Cebpb	0.31	0.43	0.26
Myelination	X89638.1	Myelin-associated oligodendrocytic basic protein	Mobp	0.35	0.18	0.12
Myelination	NM_017190.1	Myelin-associated glycoprotein	Mag	0.47	0.36	0.29
Myelination	NM_022668.1	Myelin oligodendrocyte glycoprotein	Mog	0.44	0.32	0.19
Myelination	NM_012798.1	Mal, T-cell differentiation protein	Mal	0.37	0.28	0.28
Myelination	AA945178	Signal recognition particle receptor, B subunit transferrin	Srprb Tf	0.33	0.27	0.15
Zinc ion binding	NM_012566.1	Growth factor independent 1 transcription repressor	Gfi1	0.20	0.44	0.41
Zinc ion binding	AW529624	Zinc finger protein 91	Zfp91	0.33	0.32	0.38
Actin binding	AW522439	Ermin, ERM-like protein	Ermn	0.43	0.42	0.28
Apoptosis	BG377720	Solute carrier family 5 (sodium/glucose cotransporter), member 11	Slc5a11	0.25	0.19	0.19
Apoptosis	U21955.1	Eph receptor A	Epha7	0.34	0.48	0.18
Cell Adhesion	BM391100	Mucin 4, cell surface associated	Muc4	0.43	0.36	0.27
Other	AW435010	Protein tyrosine phosphatase, non-receptor type 3	Ptpn3	0.38	0.46	0.36
Other	AF312319.1	gamma-aminobutyric acid (GABA) B receptor 1	Gabbr1	0.33	0.41	0.39
Other	NM_053936.1	Endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 2	Edg2	0.47	0.31	0.31

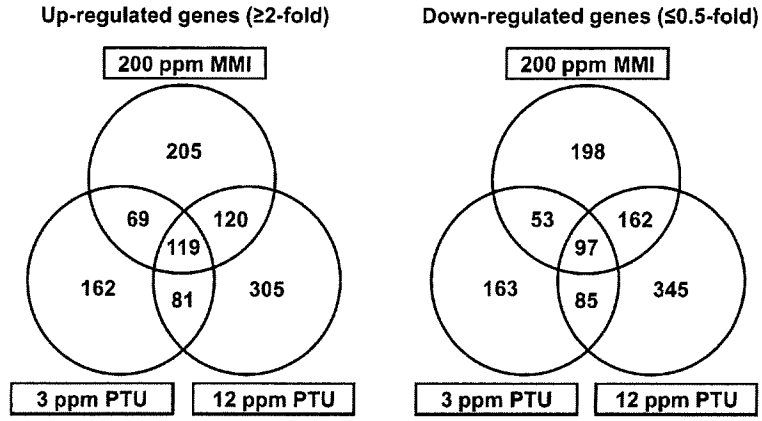

Fig. 2. Venn diagram of gene populations showing altered expression in the hippocampal CA1 pyramidal cell layer at postnatal day 20 in response to maternal exposure to propylthiouracil and/or 2-mercapto-1-methylimidazole compared with the untreated controls. (Left) Up-regulated genes (≥2-fold). (Right) Down-regulated genes (≤0.5-fold). Abbreviations: MMI, 2-mercapto-1-methylimidazole; PTU, 6-propyl-2-thiouracil.

Table 2. Validation of microarray data by real-time RT-PCR

Gene	200 ppm MMI			3 ppm PTU			12 ppm PTU		
	Microarray	Real-time RT-PCR normalized to		Microarray	Real-time RT-PCR normalized to		Microarray	Real-time RT-PCR normalized to	
		Hprt[a)]	Gapdh[b)]		Hprt	Gapdh		Hprt	Gapdh
Tacr3[c)]	7.32 ± 2.21**	4.29 ± 1.27	4.08 ± 1.15*	6.19 ± 2.19**	3.46 ± 1.42	3.76 ± 1.51*	12.49 ± 1.56**	9.23 ± 3.00**	8.81 ± 1.60**
Calb1[d)]	4.48 ± 0.66*	3.96 ± 0.74	3.67 ± 0.16	4.85 ± 2.53*	4.74 ± 2.48	4.93 ± 3.79	9.00 ± 1.85**	11.13 ± 2.13**	10.53 ± 3.26**
Slit2[e)]	3.04 ± 0.79	2.83 ± 0.90	4.08 ± 1.15*	2.62 ± 1.16	1.33 ± 0.67	3.67 ± 1.51*	7.08 ± 2.15**	4.72 ± 2.57**	8.81 ± 1.60**
Plgl1[f)]	3.10 ± 1.57	12.67 ± 5.00	11.5 ± 7.50	4.28 ± 2.88	18.33 ± 6.00	19.00 ± 9.00*	6.86 ± 2.85**	30.67 ± 5.33**	27.00 ± 8.00**
Mobp[g)]	0.35 ± 0.15**	0.6 ± 0.22*	0.52 ± 0.16**	0.18 ± 0.07**	0.24 ± 0.07**	0.24 ± 0.05**	0.12 ± 0.02**	0.18 ± 0.04**	0.16 ± 0.04**
Edg8[h)]	0.40 ± 0.11*	0.49 ± 0.16*	0.43 ± 0.13*	0.06 ± 0.05**	0.29 ± 0.10**	0.28 ± 0.08**	0.08 ± 0.07**	0.21 ± 0.07**	0.18 ± 0.03**
Cebpb[i)]	0.31 ± 0.06**	0.43 ± 0.04**	0.38 ± 0.06**	0.43 ± 0.18**	0.77 ± 0.07	0.76 ± 0.10	0.26 ± 0.04**	0.39 ± 0.16**	0.35 ± 0.22**

a) Hprt, Hypoxanthine-guanine phosphoribosyltransferase; b) Gapdh, Glyceraldehyde 3-phosphate dehydrogenase; c) Tacr3, Tachykinin receptor 3; d) Calb1, Calbindin 1; e) Slit2, Slit homolog 2 (Drosophila); f) Plgl1, Pleomorphic adenoma gene-like 1; g) Mobp, Myelin-associated oligodendrocytic basic protein; h) Edg8, Endothelial differentiation, sphingolipid G-protein-coupled receptor, 8; i) Cebpb, CCAAT/enhancer binding protein (C/EBP), beta.

Values are mean ± SD (n=4) relative to the expression level in the untreated controls. Real-time RT-PCR analysis of Hprt and Gapdh was performed in the analysis of each target gene.

*, **: Significantly different from the untreated controls at $P<0.05$ and $P<0.01$, respectively (Dunnett's multiple comparison test).

tion in response to 12 ppm PTU was approximately 2-fold higher than that with 3 ppm PTU. The number of genes showing up- or down-regulation in response to 200 ppm MMI was in between that elicited by 3 or 12 ppm PTU. One-hundred nineteen genes were up-regulated in common by MMI and PTU, with PTU showing up-regulation from 3 ppm. On the other hand, 97 genes showed down-regulation in all MMI and PTU groups. Representative genes showing up- or down-regulation in all three groups are shown in the Table 1. Among the genes listed, genes associated with nervous system development, zinc ion binding, apoptosis and cell adhesion were commonly up- or down-regulated. Genes related to calcium ion binding were found to be up-regulated and those for myelination were often down-regulated.

Real-time RT-PCR analysis: For confirmation of the microarray data, four genes that were up-regulated and three that were down-regulated in response to anti-thyroid agents were selected for mRNA expression analysis by real-time RT-PCR and the results are summarized in Table 2.

In all exposure groups, many of the expression changes were similar in the two analysis systems, except for much higher expression of *Plagl1* in all exposure groups by real-time RT-PCR as compared with findings from the microarray system.

Although we performed expression analysis of *Efna5* by real-time RT-PCR, expression values were rather low with great variability between samples, and therefore, reliable quantitative data could not be obtained (data not shown).

Immunolocalization of EphA5 and Tacr3 in the hippoc-