*2.3. Animals*

Laboratory details regarding rat strain, age of castration, age at start of dosing, day of autopsy, animal diet, and the number of animals housed per cage are summarized in Table 1. Two laboratories used Crj:CD (SD) rats castrated at 6 weeks old, and the test substances were administered 1 week after castration. One laboratory used Brl Han: WIST Jcl (GALAS) rats castrated at 6 weeks old, and the test substances were administered 2 weeks after castration. In all of the laboratories, the rats were weighed, weight-ranked, and assigned randomly to each of the experimental and control groups after they had recovered from their operation. Body weight and clinical signs were recorded daily throughout the study. Rats were provided with water and a commercial diet ad libitum. The animals were kept under SPF conditions. All animals were cared for according to the principles outlined in the guide for animal experimentation prepared by *The Japanese Association for Laboratory Animal Science*.

*2.4. Administration*

We performed each test according to the protocol proposed by the OECD (OECD 2000, 2002, 2003). Each test substance was orally administered via a stomach tube for 10 consecutive days at approximately the same time each day. A vehicle control group receiving only corn oil was used in both versions. For the antagonistic version, 0.2 mg/kg/day of TP was coadministered each day by subcutaneous injection in the dorsal region after the oral administration of each chemical. The volume of the corn oil solution containing the TP was 0.5 ml/kg. In the agonistic version, a positive control group of animals received TP injections alone. The group size in all cases was six rats. The volume of the corn oil solutions containing each of the test chemicals was 5 ml/kg. The animals were killed by bleeding from the abdominal vein under deep ether anesthesia approximately 24 hours after receiving their final dosage. The five mandatory tissues, the ventral prostate and fluid, seminal vesicle and fluid, the levator ani and bulbocavernosus muscle complex (LABC), glans penis, and Cowper's gland, were carefully dissected free of adhering fat and weighed to the nearest 0.1 mg. We also weighed the liver in 3 laboratories, and paired kidney and adrenal weights were measured in 1 laboratory.

*2.5. Statistical analysis*

We received the information from the coordinator of this Phase 3 validation after all tests were finished that the participating laboratories received pairs of the test chemicals (i.e., L and E, F and G, I and C, or K and D), so we analyzed the data using the following analytical methods between the vehicle control group and the same chemical groups in the agonistic version, and the TP group and the same chemical groups in the antagonistic version. In addition, coded A and F were nonylphenol, B and G were dinitrophenol, E and L were trenbolone, C and I were *p,p'*-DDE, and D and K were linurone. Body weight and organ weight data were analyzed by Bartlett's test for homogeneity of variance. When the variance was homogeneous at a significance level of 5%, one-way analysis of variance was performed. If a significant difference was found, the difference between the control group/TP group and each of the dosage groups was analyzed with Dunnett's test. If the variance was not homogeneous, the Kruskal-Wallis test was used. If a significant difference was found, the difference between the control group/TP group and each of the dosage groups was analyzed by the nonparametric Dunnett's test. On the other hand, differences in body weight and organ weight between the control group and the TP group, coded A or B in the agonistic version and between TP group and the group using coded H, G or F in the antagonistic version were assessed for statistical significance by the two-tailed Student's t test. For graphical presentation, the sex accessory organ data were normalized to visually compare the shapes of the responses produced by each laboratory. For this normalization, the control value was set to 100% in the agonistic study, and 100% in the TP without coded compound in the antagonistic study. Analyses of variance were performed on the data from each laboratory and for the pooled laboratory data; these normalized values were not analyzed statistically.

## 3. Results

### *3.1. Agonistic version*

*Body weights, clinical observations, and optional organ weights:* Terminal body weights in rats given L were significantly lower than in rats given vehicle alone in Labs #2 and 3, and tendency towards lowering of the terminal body weights was observed in Lab #1. No abnormal clinical signs were observed in any of the rats that were treated with each substance. The paired kidney weights in rats given substance A and TP were significantly higher than in rats given only the vehicle in Lab #3, and the liver weights in rats given A and TP were also higher than in rats given the vehicle only in Lab #2.

*Accessory sex organ weights:* The accessory sex organ weights of rats given TP only in all laboratories were higher than these of rats given the vehicle alone, confirming the reliability of this study. Almost all accessory sex organ weights and total five organs in rats given L were higher than in rats given the vehicle in all laboratories. The LABC weights in rats given E was significantly higher than in rats given the vehicle in Lab #2, but the normalized change in this organ was not apparent. Normalized weight changes of the glans penis in rats given coded L showed the weakest response among five organs.

### *3.2. Antagonistic version*

*Body weights, clinical general observations, and optional organ weights:* Two rats given I plus TP died with toxic signs such as decreasing body weight, soft feces, reddish urine and weakness at 7-10 days after the administration in Labs # 2 and # 3, respectively. The terminal body weights in rats given I plus TP or K plus TP were significantly lower than in rats given TP only in 2 laboratories. The paired adrenals in rats given K plus TP were significantly higher than in rats given TP in Lab #3. The liver weights in rats given I plus TP were higher than in rats given TP in all laboratories, and increased liver weights were also observed in rats given C in Lab # 1.

*Accessory sex organ weights:* All accessory sex organ weights of rats given H which is a positive compound, flutamide, plus TP were lower than those of rats given TP, confirming the reliability of this version. Almost all the accessory sex organ weights in rats given I plus TP and K plus TP were significantly lower than in rats given TP in all laboratories. Some accessory sex organ weights in rats given C plus TP and D plus TP were also lower than in the rats given TP. Although the LABC weight in rats given G plus TP was significantly lower than that in the TP group in Lab #2, the normalized change of this organ was not so apparent. The total of the five accessory sex organ weights in rats given I plus TP and K plus TP was lower than in rats given TP in all laboratories. The seminal vesicle weight changes in rats given I plus TP and K plus TP were most sensitive among the five organs.

## 4. Discussion

Japanese laboratories performed the validation studies of Phase 2 using methyltestosterone, vinclozolin and *p,p'*-DDE as a part of the national validation program with the result that the Hershberger assay proposed by the OECD was suggested to be a good screening assay to detect androgen agonistic and antagonistic effects (Yamasaki et al. 2003a).

We also performed the Hershberger assay using coded chemicals as part of a national validation Phase 3 as the next step for the OECD guideline process of this assay. The weights of all the accessory sex organs from the experimental animals in all the laboratories exhibited significantly the same changes in the agonistic version; almost all organ weights increased in the rats given coded substance L, and no organ showed any response in rats given coded substances A and B. We received the information from the coordinator of this

validation study after all tests were finished that a group of L and E was the same compound and a dose of L was higher than that of E, and that A and B were reported to have no agonistic properties and L and E were a weak agonistic compound. In addition, the normalized weights of all the tissues treated with coded substances in each assay fell within narrow ranges. Therefore, we think that the Hershberger assay is a good screening assay for detecting the androgen agonistic effects of chemicals. The findings that the terminal body weights in rats given coded L were depressed in all laboratories and no body weight changes were detected in rats given coded substance E in all laboratories mean that a dose of L was a toxic level and a dose of E had no observed effect. The androgen agonistic effects were detected by the administration of toxic level in this study, but weak agonistic and antagonistic properties of some weak chemicals were detected when non-toxic level doses were administered (Yamasaki et al. 2003a, 2003b).

In the antagonistic version, almost all the sex accessory organs decreased in rats given coded substances I plus TP and K plus TP in all laboratories compared with each organ weight in the rats given TP only, and some organ weights also decreased in the coded substance C plus TP and D plus TP groups. No changes were detected in rats given coded substances F and G. These findings demonstrate that coded substances I, C, K, and D had antagonistic properties and coded substances F and G had no antagonistic properties. We accepted the information; substances F and G were negative compounds, I, C, K and D were weak antagonistic compounds, and H was a positive control compound, flutamide; the groups of substances I and C, or K and D were the same compound, and dose levels of I and K were higher than those of C and D. We also received the information that C and I were *p.p'*-DDE and D and K were linurone. The ventral prostate and glans penis in Lab #1, the seminal vesicle and LABC in Lab #2 and the ventral prostate, LABC and Cowper's glans in Lab #3 were significantly affected in the rats given coded substance C plus TP. In addition, the ventral prostate in Lab #1 and seminal vesicle in Labs #2 and #3 were significantly affected in rats given coded substance D plus TP. The differential effects across laboratories were observed in rats given coded C plus TP and D plus TP. We found that the most sensitive organ among the five accessory sex organs was the prostate and/or seminal vesicle in our previous validation Phase 2 study, and in the Hershberger assays using various chemicals (Yamasaki et al. 2003a, 2003b). The ventral prostate and/or seminal vesicle were responded with or without significant differences in rats given coded substances C plus TP and D plus TP, so we determined that coded C and D have androgen antagonistic properties. On the other hand, the LABC weight in rats given coded substance G plus TP was significantly lower than that in the TP group in Lab #2, but the normalized change of this organ was not so apparent. Therefore, the Japanese data in this study demonstrated that the Hershberger assay is considered to be a good screening assay for detecting the androgen antagonistic effects of chemicals. The findings that some animals died in rats given coded substance I and decreased body weights were detected in rats given K and I, and the liver weights increased in rats given I mean the coded substances I and K were at a toxic dose level. In addition, the liver weights increased in rats given C in one laboratory, so a dose of C may be at a toxic level. The general toxicity is considered to be important for this assay, because a 10% change in terminal weight is suggested to affect some Hershberger assay endpoints (Marty et al. 2003).

In conclusion, we performed the OECD validation study Phase 3 using coded chemicals. All five accessory sex organs responded with statistically significant changes in weight within a narrow window in the agonistic and antagonistic versions, and no false positive or false negative results were observed in this study. Therefore, the Japanese studies support the Hershberger assay as a reliable and reproducible screening assay for the detection of androgen agonistic and antagonistic effects.

## 5. Acknowledgments

This study was supported by the grant by the Ministry of Economy, Trade and Industry, the Ministry of Health, Labour and Welfare, and Ministry of the Enviroment in Japan.

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別添 5

III. 研究成果の刊行に関する一覧表

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# 書　籍

SPRINGER REVIEWS

井上 達／井口泰泉 編

# 生体統御システムと内分泌撹乱

Springer
シュプリンガー・フェアラーク東京

# 2. 内分泌撹乱化学物質の神経幹細胞分化に及ぼす影響

五十嵐勝秀・菅野 純

## 2. 1. 要旨

神経幹細胞は自己複製能と多分化能，すなわち神経細胞，グリア細胞(アストロサイト，オリゴデンドロサイト)といった神経系を構成する3種類のすべての細胞に分化する能力をもつ幹細胞である．神経幹細胞は胎児の脳形成および発達を司るのみならず，成熟後の脳にも存在し神経新生を行っている．一方，脳内にはエストロゲン受容体を始めさまざまな核内受容体が発現していることから，内分泌撹乱化学物質の脳に対する影響が研究されているが，神経幹細胞に標的を定めた研究例は少ない．本章ではまず神経幹細胞について概説し，次に核内受容体，特にエストロゲン受容体と神経幹細胞のかかわりに関する研究について紹介し，われわれがジエチルベストロール(DES)を用いて得た研究結果にもふれる．

## 2. 2. 神経幹細胞と神経幹細胞分化を誘導する因子

神経幹細胞は哺乳類の中枢神経系に存在する幹細胞で，未分化な状態を保ったまま増殖する自己複製能と，複数の種類の細胞(ニューロン，アストロサイト，オリゴデンドロサイト)に分化する多分化能を併せもつ細胞である(図2・1)．発生期の脳には神経幹細胞が多数存在し，脳の形成・発達を担っているが，成熟後の脳にも神経幹細胞は見出され，神経新生を行い，脳機能維持に寄与していると考えられている．神経幹細胞の存在が初めて実験的に確認されたのは，Weissらによる，

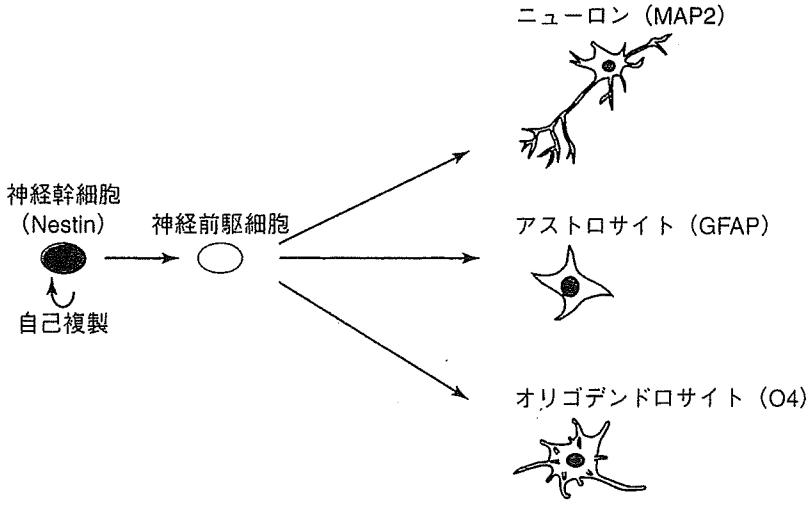
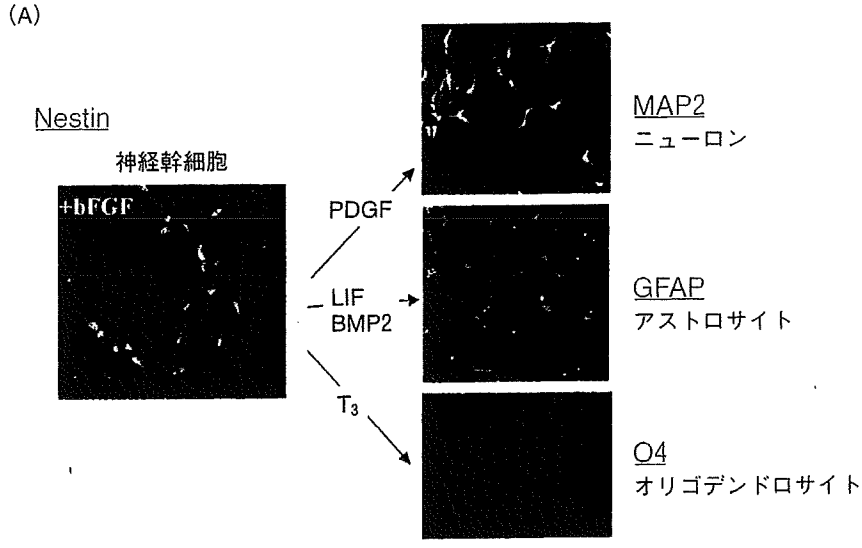



図2・1 神経幹細胞の自己複製と分化．神経幹細胞は，未分化な状態を保ったまま自己複製し，神経系を構成する3種類の細胞(ニューロン，アストロサイト，オリゴデンドロサイト)に分化する幹細胞である．おのおのの細胞には特異的なマーカータンパク質が同定されている(かっこ内に示した)．

“ニューロスフェア(neurosphere)法”という培養法を用いた研究によってである(図2・3参照)[1]. ニューロスフェアは，脳神経組織細胞を単一細胞化した後，basic fibroblast growth factor(bFGF)とepidermal growth factor(EGF)の存在下浮遊培養することにより，1週間程度の後に形成される単クローン性の細胞凝集塊である．ニューロスフェアを構成する細胞は，神経幹細胞に特異的な中間径線維タンパク質であるNestin陽性の細胞が主で，形成されたニューロスフェアを再度分散し浮遊培養すると，単一細胞から再びニューロスフェアが形成されることから，これらの細胞が自己複製能を有していると考えられている．また，ニューロスフェアを接着性の容器に移して血清などの存在下で培養するとニューロン，アストロサイト，オリゴデンドロサイトを産生する多分化能を示す．

神経幹細胞からの分化を誘導する細胞外因子については，ニューロンへの分化を促す因子としてplatelet derived growth factor(PDGF)，アストロサイトへの分化を促す因子としてleukemia inhibitory factor(LIF)とbone morphogenic protein(BMP)，オリゴデンドロサイトへの分化を促進する因子として甲状腺ホルモン($T_3$)が同定されている[2](図2・2A，口絵7参照)．これらのなかで特に，LIFとBMPによるアストロサイトへの分化促進作用に関して，それぞれの下流で活性化される転写因子Stat3とSmad1が，アストロサイト特異的遺伝子の1つであるglial fibrillary acidic protein(GFAP)遺伝



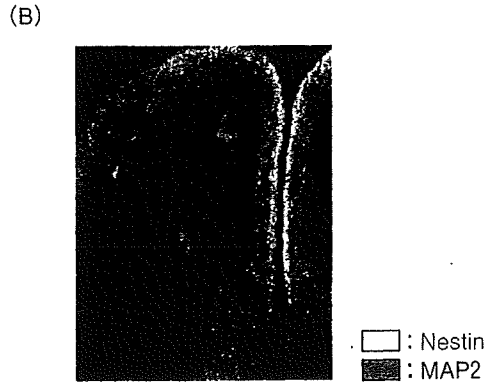




図2・2　神経幹細胞分化を誘導する因子と胎児脳におけるNestin，MAP2の発現．(A)*in vitro*培養下，Nestin陽性の神経幹細胞は特定の細胞外因子に反応し，各種細胞に分化する．PDGFはニューロンへの分化，LIFやBMP2はアストロサイトへの分化，甲状腺ホルモン$T_3$はオリゴデンドロサイトへの分化を促進する．(B)胎生14.5日の脳におけるNestin，MAP2の発現．側脳室周囲領域はNestin陽性の細胞(中央の陰の部分)で占められ，皮質側はMAP2陽性細胞(外縁部)で占められている(口絵7)．

子のプロモーター領域上でp300タンパク質を介した複合体を形成し，直接的にGFAP発現を誘導することが明らかにされている[3]．LIFとBMPは相乗的にアストロサイト分化を促進するが，その相乗効果を説明しうる分子実体がこの転写因子複合体なのである．

以上のように，神経幹細胞は細胞外因子によって分化制御されうる細胞であり，細胞外因子のシグナルは最終的に神経幹細胞内の特定の転写因子群に到達する．一方で，内分泌撹乱化学物質は，転写因子である核内受容体が細胞内標的である．このことからも，内分泌撹乱化学物質が転写制御機構を介し，神経幹細胞に何らかの影響を及ぼすことが予想される．

## 2．3．胎児神経幹細胞と成体神経幹細胞

現在，発生初期の神経誘導によって形成される神経板・神経管を構成する神経上皮細胞が，神経幹細胞そのものであると考えられている．

発生初期に神経幹細胞は増殖を繰り返すが，胎児にはこの時点ですでに頭部から尾部にかけてHoxをはじめとする転写因子群の発現の組み合わせパターンを伴う領域化が起こっており，神経幹細胞自体もその存在部位によって異なる性質を獲得している[4,5]．すなわち，神経幹細胞はヘテロな集団であり，自己複製能と多分化能を有するという点では共通の性質をもつが，発生初期にすでに個性をもっており，増殖速度も異なっていると考えられている．また，胎児期の神経幹細胞には通常予想されるのとは異なる性質がある．すなわち，神経幹細胞は始めはニューロンにのみ分化する能力を有しているにすぎず，そのため発生初期はニューロンが優先的に産生される時期が続く．産生されたニューロンは定まった位置に移動し，神経核や層構造を形成する．発生中期から後期にかけて神経幹細胞はアストロサイトやオリゴデンドロサイトといったグリア細胞にも分化する能力を獲得し，ニューロンに加えてグリア細胞を産生し，脳の形成発達が進む．このような，発生に伴って多能性を獲得していくという性質が胎児神経幹細胞の特徴である[6]．

一方，神経幹細胞は成体の脳においても存在することが示されている[7-11]．すなわち，海馬をはじめとする成体の脳のさまざまな領域から，bFGF，EGFに反応し，ニューロスフェアを形成する細胞が同定されている．神経幹細胞の局在については，脳室を直接取り囲む上衣層(ventricular zone，VZ)もしくはVZのすぐ内側の上衣下層(subventricular zone，SVZ)に存在するという2つの説が唱えられている[12,13]．いずれにせよ，成体の神経幹細胞は脳室の周囲領域に存在していると考えられている．しかし，成体の神経幹細胞と胎児神経幹細胞とが，どの程度異なった性質を有しているかはほとんど明らかになっていない．

## 2. 4. 核内受容体の神経幹細胞における機能

内分泌撹乱化学物質の標的はそのほとんどが核内受容体である．よって，神経幹細胞で発現し機能している核内受容体は内分泌撹乱化学物質研究において重要な対象と考えられる．しかし，これまで神経幹細胞と核内受容体とを関連づけて研究された例は少ない．

そのなかで最近，オーファン受容体の1つTLXが成体神経幹細胞の未分化性の維持に必須であるという報告が，米国ソーク研究所のEvansらのグループによりなされた[14]．マウスにおけるTLXの発現は胎生初期から認められ，中期にピークを迎え，出生直後にはいったん消失する．その後発現は上昇し，成体の脳では高い発現が保たれる．TLXのノックアウトマウスを用いた解析により，TLXを欠く成体の神経幹細胞は増殖せず，TLXを再度導入することで増殖能を回復すること，TLXは転写抑制因子として働き，アストロサイト特異的遺伝子であるGFAPの発現を抑制することで神経幹細胞の未分化性を保っていることが示された．

TLXはオーファン受容体でありリガンドが不明であることから，この結果をすぐに内分泌撹乱化学物質と結びつけて考えることはできないが，核内受容体による神経幹細胞機能の制御の例として注目される．

エストロゲン受容体については，イタリアのMaggiらのグループによるヒト神経芽細胞腫細胞株SK-N-BEを用いた研究がある．TGFαによる細胞増殖刺激が，エストロゲン受容体α型を強制発現させることで分化刺激に変換されることが報告されている[15]．この場合，発現したエストロゲン受容体α型は，TGF-αにより活性されたStat3と結合し，分化を促進するとのモデルが提唱されている．また，Maggiらは2004年のKeystone Symposiaにて，エストロゲンは成体脳に作用し，脳室周囲領域の細胞によるBrdUの取り込みを促進すること，その領域でエストロゲン受容体α型依存的にProthymosin αの発現を上昇させること，Prothymosin αのアンチセンスRNAを脳室内に投与してその発現を抑制するとBrdUの取り込みが低下し，エストロゲンによって誘発されるロードーシス反応(雌の受容反応)が抑制されることを報告した．彼女らは，成体の神経幹細胞はエストロゲンに反応し増殖すると考えている．さらにTanapatらにより，エストロゲンはラット成体脳海馬における神経新生を促進することも報告されている[16]．すなわち，雌の卵巣を除去すると海馬において新生される神経幹細胞の数が減少し，エストロゲンを投与すると回復することが示されている．加えて，Brannvallらは，神経幹細胞にエストロゲン受容体α型，β型ともに発現しており，分化能の制御にかかわっているとの報告をした[17]．しかし，彼らは神経幹細胞マーカーの発現を検討していないことから，その報告ではエストロゲン受容体が神経幹細胞を含む領域で発現していることを示したにとどまっている．

次に神経幹細胞と核内受容体，特にエストロゲン受容体に関するわれわれのデータを紹介したい．