

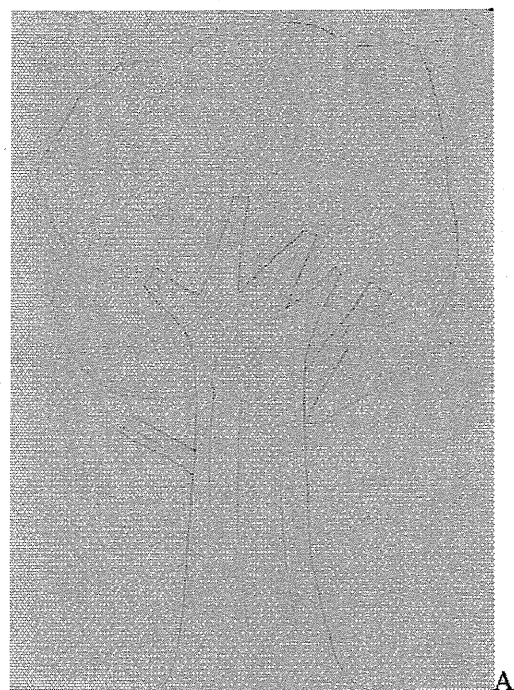
Figure 2. Tree drawing test drawing of the patient at Stage I

Tree drawing test drawing of the patient at Stage I is shown. Note that the branches are cut off in the middle. This feature is consistently observed in the rest of her drawings. The rigid crown shape and the solid and strong lines are also characteristic.

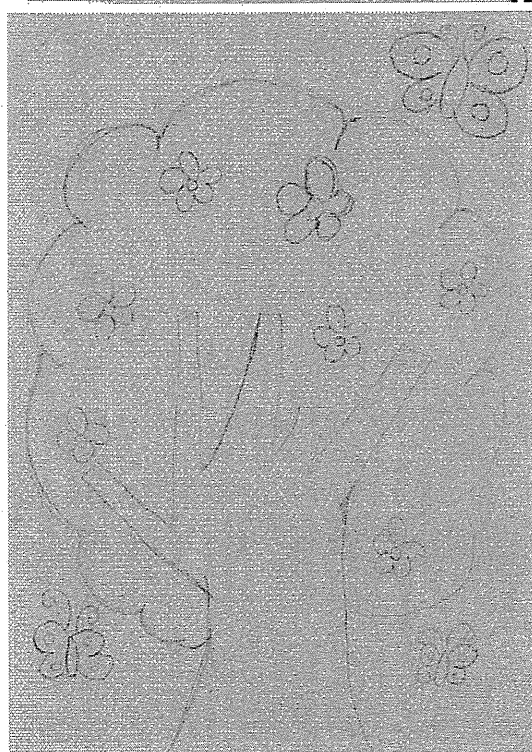
The Tree Drawing Test

Figure 2 shows the patient's drawing at Stage I. The form of her drawing was interpreted as defensive to stimuli from the outer world (outlining of the crown is rigidly shut) and emotionally shut off from relationships with others (the branches are all cut off in the middle), which are both considered to represent a pathologically low energy state. The lines of drawing were consistently solid and high in strength during all stages, which was thought to represent the inherent individuality of her obsessive tendency.

Figures 3A and 3B were both obtained during Stage II, the first hospitalization period. On admission (Figure 3A), the crown was drawn with discontinuous lines in the tree drawing test, suggesting her susceptibility to the outer world. The branches were still the same, having the cut-off-in-the-middle shape, suggesting her emotionally shut-off state. In addition, the crown was shaded, which was thought to represent her depressive state. The size of the crown was reduced and a branch grew beneath the crown, which was considered representative of her psychological regression. On discharge after approximately a month of hospitalization, her drawing (Figure 3B) still showed signs of defensiveness and emotional shutting off. However, the crown was enlarged and was no longer painted, and no branches were seen beneath the crown. These observations suggested that her psychological regression had ended. Instead, the drawing showed flowers and butterflies with a trunk of wide width, which was thought to represent her self-display and magnified self. The lines of the drawing are solid and very strong, similar to those of previous drawings.

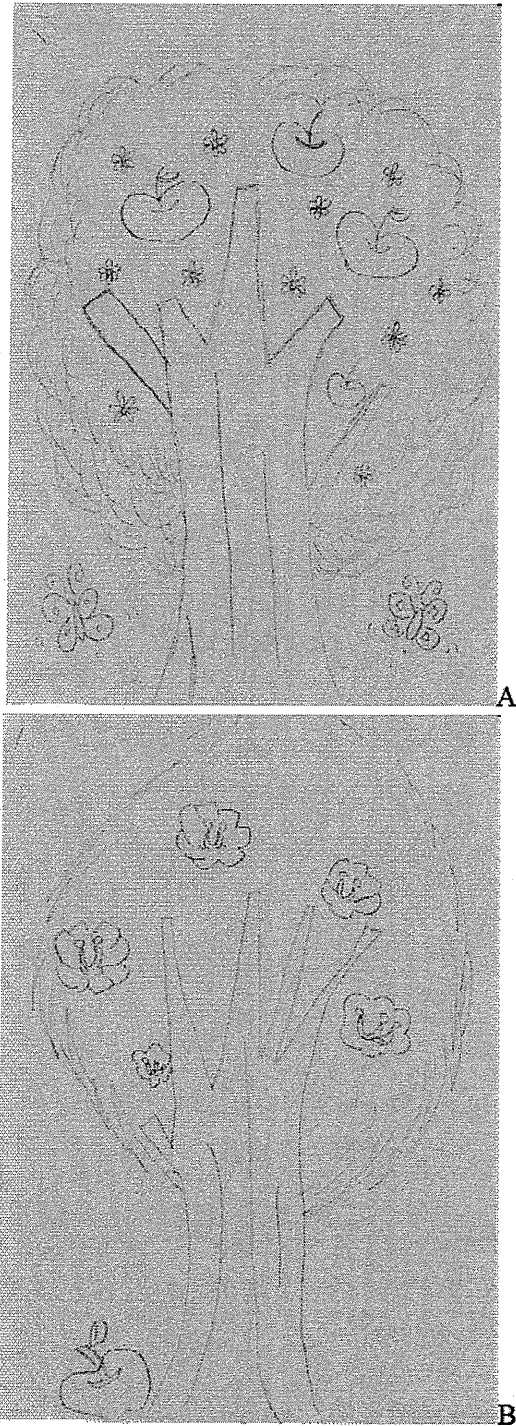


A

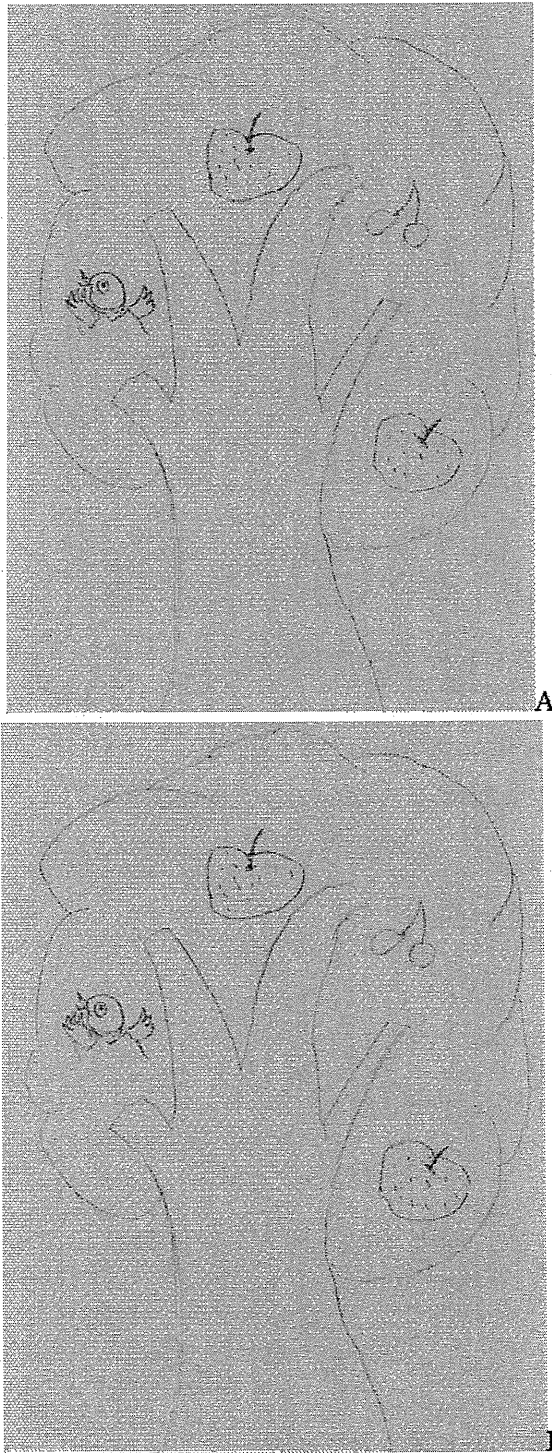


B

Figures 3A and 3B. Tree drawing test drawings of the patient at Stage II. Tree drawing test drawings at Stage I, on admission (Figure 3A) and on discharge (Figure 3B) following the patient's first hospitalization period are shown. Note that the defective crown size and form seen on admission have improved by discharge.



Figures 4A and 4B. Tree drawing test drawings of the patient at Stage III. Tree drawing test drawings earlier (Figure 4A) and later (Figure 4B) in Stage III are shown. The patient was obsessed banana ingestion, and her condition gradually deteriorated during this period. Note that the outline of the crown was drawn differently, and later, the crown protruded from the paper.



Figures 5A and 5B. Tree drawing test drawings of the patient at Stage IV. Tree drawing test drawings at Stage IV on admission (Figure 5A) and on discharge (Figure 5B) following the second hospitalization period are shown. Note that on admission, the form of the drawing seems collapsed and the crown has a discontinuous outline, showing loss of energy.

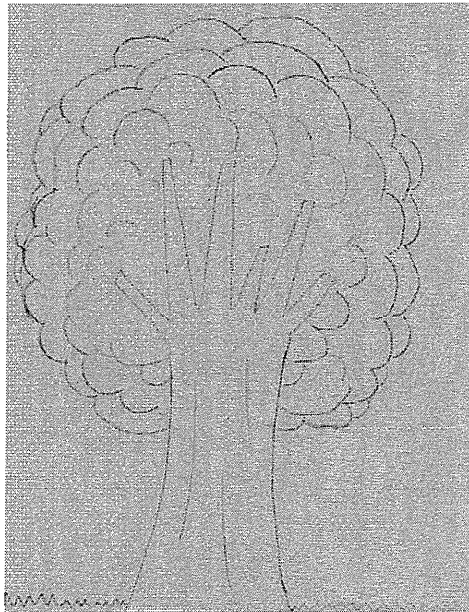


Figure 6. Tree drawing test drawing of the patient at Stage V.

Tree drawing test drawing at Stage V is shown.

Note the formation of the crown with lines rising one by one from the inner side of the tree, as if showing the patient's effort to resist her bursting inner-impulse.

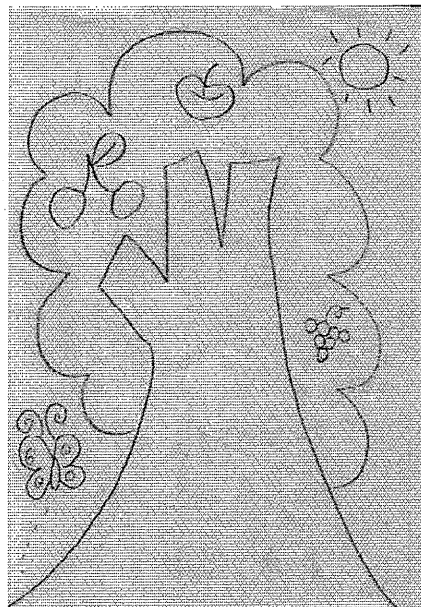


Figure 7. Tree drawing test drawing of the patient at Stage VI.

Tree drawing test drawing at Stage VI is shown. After remission of the obsessive banana ingestion, the bizarre observations seen in the Tree drawing test drawing at Stage V have completely disappeared and the drawing now resembles figures 3B and 5B, from the earlier post-treatment periods.

During Stage III, the period when the patient's obsessive banana ingestion began and continued, the tree drawing test was performed twice and an obvious change in the crown form was observed. The outline of the crown was drawn fluffy with repeated lines in the earlier drawing of Stage III (Figure 4A), which was deformed into more sharp-pointed and discontinuous form in the later drawing (Figure 4B). These observations were interpreted to mean that as her susceptibility to outer stimuli grew and she needed to pile up the lines to be more defensive. Later on, she became irresistibly hypersensitive and more aggressive. This time, the crown protruded out from the paper, representing her acting-out state.

During Stage IV, the second period of hospitalization that occurred when her critical emaciation had progressed as a result of her obsessive banana ingestion, the tree drawing test was performed twice (Figures 5A and 5B). In Figure 5A, the tree drawing test was performed upon admission. The patient's drawing showed a wide trunk, branches beneath the crown (regression), and a discontinuous outline of the crown. It seemed she no longer had the energy to shut the crown through repeated drawing; the single discontinuous line can easily let the outer stimuli invade into her inner-self. On discharge after the second hospitalization period, the form of the tree drawing test (Figure 5B) showed slight improvement, with a wide trunk and fruits that represent self-display and magnified self.

Generally speaking, to this point, her psychological energy had been decreasing in tandem with the stage progression. However, at Stage V, when her obsessive eating was at a maximum and her blood dopamine concentration was elevated as high as 180 ng/ml, her drawing showed drastic changes. Figure 6 shows her drawing at this stage. The form of the crown was completely changed, with lines rising one by one from the inner area of the tree, as if showing the effort of the patient's resistance to her bursting inner-impulse. For the first time, a horizon appeared in her drawing as a notched line, representing a decrease in her anxiety and an apparent stabilization (in her subjective sense, although she was not healthy). The lines were drawn smoothly with no disconnection, showing her full of energy without anxiety or depression. These changes were totally inexplicable and unexpected based on her physical state, which was characterized by body weight as low as 30 kg, indicating that she was critically ill with anorexia nervosa.

At Stage VI, the bizarre observations seen in the drawing at Stage V had completely disappeared, namely, the drawing showed the pattern that had originally been observed in the patient (Figure 7). The form of the crown was smooth, with a continuous outline with no rigidity, indicating that her defensiveness and susceptibility to the outer stimuli were decreased in her current psychiatric state. The extremely wide trunk (especially at the bottom) and fruits/butterfly showed that her self-display and magnified self-tendency still exist, suggesting that she was not completely healthy but was seemingly back to her original psychiatric state as a result of stopping banana ingestion and the consequent decrease in her blood dopamine concentration.

The STAI

The STAIs were performed at Stages III, IV, and V. The STAI-state anxiety score was 55, 68, and 46, and the STAI-trait anxiety score was 47, 58, and 49, respectively, at each stage. At Stage V, the patient's anxiety levels, which had been rising along with her illness

condition suddenly decreased, apparently showing that the patient "did not feel" anxious anymore during this period. These findings were similar to the results of the tree drawing test.

The Ego State

The patient's ego states were examined at Stage I and Stage V. At Stage I, low energy was observed in the egogram, and low CP (Critical Parent) and FC (Free Child) were observed along with high AC (Adopted Child). These results were interpreted to indicate low ego state, low self-assertion, and emotional repression with high law-abiding tendency. This observation was consistent with her tree drawing test and the diagnosis of restricting anorexia nervosa. In contrast, at Stage V, high energy was observed in the egogram. Her ego state showed a result that was the complete inverse of that in Stage I, with high-rising CP and FC, and an obvious decrease in AC. At this stage, the patient "feels" high ego energy, high self-assertion, and emotional elevation with low law-abiding tendency, and is seemingly full of vigor. Again, this observation did not contradict the results of the tree drawing test and STAI at this stage, suggesting transitory and unexpected psychological alteration had occurred at Stage V.

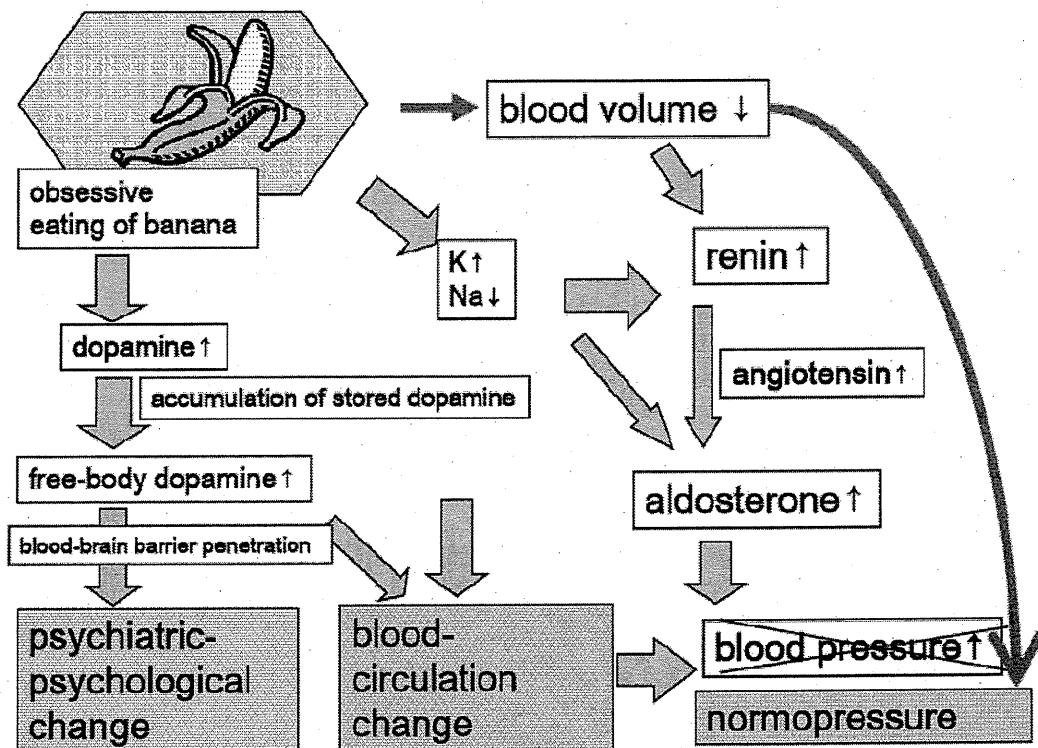


Figure 8. The summarized feature of the case with obsessive eating of banana is shown. The increase of blood potassium and dopamine induced by unusual consumption of banana was considered to be responsible for the pseudoaldosteronism and the psychiatric-psychological change in the patient.

DISCUSSION

The patient followed an unusual course of restricting anorexia nervosa, which was considered to be related to her long period of obsessive and restricted banana ingestion. The summary points of the case is shown in Figure 8. Bananas are known to be a dopamine-rich food. Approximately 0.72 mg of dopamine is contained within 100 g (approximate weight of the edible portion of one banana) of ripe banana fruit, and 100 g of unripe banana fruit contains as much as 10 mg of dopamine. Moreover, 100 g of ripe banana skin and thread contain 235 mg of dopamine and 100 g of unripe banana skin and thread contain approximately 1940 mg of dopamine (Kanazawa and Sakakibara, 2000). Dopamine is absorbed in a sulfate conjugated form from the digestive tract, and normally the free dopamine level is not affected by the dose of ingested dopamine (Davidson et al 1981; Dumne et al 1983).

The patient showed some level of hyperdopaminemia from the beginning of her illness course, which is consistent with previous reports of dopaminergic neuronal hyperactivity in anorexia nervosa (Devesa et al 1988; Kaye et al 1999). However, the dramatic increase in her whole blood dopamine concentration, which was observed immediately after she began excessive ingestion of bananas, cannot be explained simply based on the pathogenesis of anorexia nervosa. Rather, considering the fact that the patient preferred unripe bananas to ripe ones and often ate the skin and thread of the banana as well, her daily ingestion of dopamine must have increased significantly. That increase may eventually have led to an increase in free dopamine (0.47 ng/ml, compared with the normal range in the Japanese population of <0.03 ng/ml), without a corresponding increase in epinephrine or norepinephrine, most likely because banana contains particularly large amounts of dopamine, but relatively smaller amounts of epinephrine and norepinephrine. We suspect that in the case of this patient, sulfate-binding dopamine became oversaturated by daily ingestion of large amounts of banana, which led to the resulting increase in free dopamine.

In addition to hyperdopaminemia, the patient demonstrated hyperkalemia and pseudoaldosteronism. Bananas are also known to be rich in potassium, containing 330–400 mg of potassium in 100 g of the edible portion of the fruit (Kasuga A, 2008). Hyperkalemia, along with hyperdopaminemia, is considered to be the leading cause of pseudoaldosteronism. An obvious increase in blood renin and angiotensin was observed, but her blood pressure remained normal throughout the course of her illness, probably because of her absolute hypovolemia.

In the initial tree drawing test and egogram performed prior to the onset of obsessive eating, the patient showed low energy and ego states, with defensiveness, low self-assertion, and a strong protective tendency, which were all reasonably explained by the psychopathology of anorexia nervosa and essential obsessive personality of the patient (Mizuta et al 2002). However, in the tree drawing test, egogram, and STAI performed after two years of obsessive ingestion of only banana, the opposite results were observed; high energy and ego states, with an extreme internal impulsiveness and low anxiety and high self-assertion. Given that this alteration in psychological state occurred with simultaneous hyperdopaminemia and disappeared along with remission of the obsessive banana ingestion, it is possible that the extreme increase in her blood dopamine level resulting from obsessive

banana ingestion was responsible for saturating binding proteins and increasing free dopamine, which affected her psychological state.

Her obvious aggression and inner-impulse, in accordance with the increase in whole blood dopamine, suggest that the brain dopamine concentration was also affected by the obsessive and restricted ingestion of banana. Dopamine is well known to have poor blood-brain barrier penetration because of physiological blockade caused by its high polarity. However, a recent study revealed that blood-brain barrier penetration of dopamine could be increased in a dose- and time-dependent manner by continuous arterial infusion of dopamine (Martel et al 1996). Thus, it seems reasonable to assume that blood-brain barrier penetration by dopamine was modified in the patient, who experienced hyper-free dopaminemia, as a result of lengthy and excessive ingestion of banana.

Nutrient imbalance resulting from deficiency of vitamins and minerals such as calcium, iron, zinc, phosphate, and magnesium may be observed in eating disorders, and this imbalance may cause skin disease, endocrinological disorder, or osteopenia (Seidenfeld et al 2004). Zinc is an important brain nutrient, acting as a neuromodulator/neurotransmitter mainly in the cortex in relation to glutamnergic neurons (Harrison and Gibbons, 1994; Takeda et al 2000). Deficiency of zinc may be related to cognitive disorder or depression (Meunier et al 2005). However, no clear link to eating disorders has yet been proven, and no sign of zinc deficiency was observed in the present case.

The case of our patient is noteworthy as an extreme example of how ingested banana directly affects the biochemical and psychic state. The patient showed strong and irrational obsessive behavior with banana and exercise compared to patients with regular anorexia nervosa. Since abnormal eating habits are common in patients with anorexia nervosa, it is necessary to identify detailed eating habits of anorexia nervosa patients to avoid the cumulative effects of a particular food on their physical and psychic state.

ACKNOWLEDGMENTS

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自閉症の動物モデル

—— 胎生期の化学物質ばく露による情動・認知行動への影響解明のための動物モデル

成田 正明

【要旨】 認知メカニズムを解明する目的で筆者らは自閉症に焦点を当て、モデル動物を作成して研究している。様々な自閉症モデル動物が提唱されているが、筆者らはヒトでの疫学的事実に着目し、妊娠ラットにサリドマイドやバルプロ酸を投与する方法で解析を進めている。これまでにセロトニン神経系の異常、行動異常などを報告してきた。本稿ではこれらについて最近の知見も含め述べるとともに、筆者が平成 21 年度より研究代表者として推進中の厚生労働省研究班「化学物質の胎内ばく露による情動・認知行動に対する影響の評価方法に関する研究」についても触れていきたい。

はじめに

自閉症は、対人関係の障害、コミュニケーションの障害、こだわりなど、人との関わりを主症状とする、先天的な脳の機能障害に基づく発達障害である。しかし「先天的」とされながら、胎生期のどの時期に、どういうことが原因で（遺伝的因子、ウイルス感染、薬剤・化学物質）、どんな機能障害が脳におきているか、はわかっていない。その症状からの類推により、発症には神経伝達物質セロトニン系の異常が根底にあると考えられているが、詳細は不明のままである。本症の有病率は現在では 150 人に一人と、30 年前に比し数十倍に増加している。これは単に診察医の診断技術の向上だけでは説明がつかないほどの増加であり、ここ数十年の妊婦をめぐる環境の変化、薬物（含アルコール）・環境ホルモン・ストレスなどに起因するとするのが自然である。2005 年発達障害者支援法が施行され発達障害者に対し正しい理解と対

応が求められており、その病態の解明は社会的にも急務であるといえる。

疾患の病態解明にはモデル動物作成が欠かせない。これまで様々な手法で自閉症モデル動物の作成が試みられてきた。筆者らは、ヒトで起こった疫学的事実を妊娠ラットで忠実に再現することで、「自閉症モデルラット」を作成し解析を行ってきた¹⁻⁴⁾。即ちサリドマイドを内服した妊婦から生まれた児に自閉症児を高率（通常の頻度の 30 倍）に合併したという疫学的事実⁵⁾に着目した。サリドマイドは胎生期のばく露により四肢の奇形を引き起こす胎芽病としてよく研究されているが、興味深いことに、妊娠のある特定の時期にサリドマイドを内服した母親からの児に高率に自閉症を発症することがわかった。しかもこの自閉症を合併したサリドマイド児には四肢の奇形は見られない一方、外耳奇形・難聴を伴っていたという。このことはサリドマイドによる自閉症併発は、受精後 20-24 日のサリドマイドばく露したことを意味する。言い換えれば、この時期における脳神経

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系の発生発達のメカニズムを詳細に解明できれば、自閉症を引き起こすような脳の初期発生異常のメカニズムを解明できるのではないかと考えるに至った。

I. 自閉症の原因、及びこれまでに報告されてきた自閉症モデル動物

自閉症の原因としては、遺伝的因子、胎内感染症、化学物質（薬物・毒物）などがある。詳細はここでは触れないが、その発症に遺伝的因子が大なり小なり関与している可能性は否定できない。筆者の診療経験からもそう感じ取れる。近年、copy number variation 仮説も提唱されてきている。しかしながら spectrum として heterogeneous な症候を持つ自閉症の病態を、単一の遺伝子異常で説明するのは困難である。先天性感染症も指摘されているが、これらもやはり妊娠中のどの時期の感染が critical なのか、virus 種に specific なのかは明らかでない。また妊婦の抗てんかん薬バルプロ酸などの薬物、アルコール、その他の化学物質の胎内ばく露も自閉症発症原因になり得るとされる。化学物質の胎内ばく露を巡っては、有機水銀摂取なども懸念事項であり、妊婦の魚介類摂取許容量が見直されるなども関連しているといえる。これらに基づいてさまざまな自閉症モデル動物が作成されてきた⁶⁻¹⁴⁾。

II. サリドマイドを用いた自閉症モデルラット——ただしサリドマイド胎芽病でみられる先天奇形と、サリドマイド自閉症とは、胎内でのばく露時期が異なる全く別の病態である

筆者らはサリドマイド薬害被害者に、情動・認知行動の一つである自閉症患者が多数発生したことに着目し、ヒトで発生したサリドマイド胎内ばく露による自閉症発症の過程を妊娠ラットでできる限り忠実に再現した。この方法を用いれば前に述べた自閉症の原因（複数かも知れない）が解明

されるのを待つことなく自閉症モデル動物が作成でき、そのモデル動物解析を通じて自閉症の本態に迫ることができることになる。自閉症スペクトラムは広汎にわたるため、もちろんこの方法で作成した自閉症モデル動物がヒトに発症している全ての自閉症と同一の病態を反映しているとは考えられず、また言うまでもなくヒトとラットという種の違いも十分考慮しなければならないが、本法で作成した自閉症モデルラットを詳細に解析することでヒト自閉症の病態解明、治療法模索につながり得る可能性は十分期待できる点で、「モデル動物」としての役割を持ち得る。

サリドマイドは1957年10月、旧西ドイツで発売された催眠薬である。当初は「安全な」薬として妊婦を含め広く用いられ、まもなく日本でも製造が始まった。ところが妊娠初期の妊婦が内服すると児に短肢症などの障害がでることがわかり回収され、悪魔の薬として市場に出回るとは一旦なくなった。しかしその後サリドマイドは多発性骨髄腫をはじめ癌、自己免疫疾患の治療薬として有効である報告が出てから世界的に使われ始めている。サリドマイド薬害の症状は短肢症がよく知られているが、特に本邦ではあまり知られていない合併症に「自閉症」がある。Stromlandらによれば受胎20-24日に経胎盤的にサリドマイドにばく露されると通常に比し、高率に自閉症を発症するという⁹⁾。即ち受胎20-24日頃における脳神経系の発生発達のメカニズムが詳細に解明できれば自閉症発症のメカニズムが解明できる可能性がある。

ヒトで受精後20-24日はラットでは妊娠9日前後に相当する。そこで私たちは以上に述べたヒトで起こった疫学的事実を、動物を用いてできるだけ忠実に再現してみることを試みた。即ち、様々な妊娠日齢の妊娠ラットを準備する。それら妊娠ラットに5%アラビアゴムに懸濁したサリドマイドを無麻酔下でゴム製カテーテルを用いた1回強制経口投与を行なった。そのまま妊娠を継続さ

せ、その後出生した仔を生化学的、形態学的、行動学的にと様々な観点から解析した。自閉症発症に神経伝達物質セロトニン系の異常の関与については未だ論議の分かれるところであるが、自閉症病態を探るための数少ないバイオマーカーのひとつではある^{15,16)}。そこでまず上述の方法で作成した仔ラットでの、セロトニンをはじめとするモノアミンの濃度を測定した。すると妊娠9日目にサリドマイドを投与した群にのみ、海馬セロトニン濃度及び、前頭葉皮質でのドーパミン濃度の有意な上昇がみられた。さらに興味深いことにこのラットでは血中セロトニン値も正常に比して有意に上昇していた。血中セロトニン値の上昇はヒト自閉症患者においても1/3程度見られる所見であり興味深い。ラットの妊娠9日目はヒトでは受精後20-24日目に当たり、妊婦のサリドマイド服用でもっとも多く自閉症児が生まれたとされる時期に一致する。同様の所見はやはり妊娠中に服用すると児に自閉症が発症すると報告されている¹⁷⁻¹⁹⁾。抗てんかん薬バルプロ酸を用いた実験でも得られた。そこで筆者らはこの妊娠9日目にサリドマイドまたはバルプロ酸を投与して得られた仔ラットを“自閉症モデルラット”として報告してきたものである。

自閉症に特異的な形態学的・病理学的所見、病態解明に密接に結びつくような所見はないといってよい。前述のごとく本モデルラットで脳及び血中セロトニン異常をみとめたため、形態学検索を行なうにあたってまずセロトニン神経系に焦点を当てた。即ちセロトニン神経の起始核である縫線核（ここでは背側縫線核）の発達の異常の有無を調べるため、縫線核のセロトニン陽性細胞数を、自閉症モデルラットとコントロールラットとで調べた。背側縫線核は頭尾方向に細長い構造を持つ。背側縫線核の中央付近では両ラットで差はほとんど見られなかったが、吻側尾側では大きな差があった。即ち正常コントロールでは縫線核セロトニン陽性細胞は吻側では多く尾側に向かうにつ

れ少なくなるのに対し、サリドマイド投与、バルプロ酸投与いずれの方法によって作成した自閉症モデルラットでは、正常コントロールとは反対に、尾側にむかうにつれて増多していた。この所見を神経発生学的見地から解釈すると以下ようになる。

セロトニン神経系は、ドーパミン神経系と並んで、もっとも初期に発生を始める神経系のひとつである。その幹細胞は、ラットにおける胎生(E)9に、ラット中脳と後脳の境界領域(isthmus)にすでに存在することが知られている。この細胞は、このisthmus付近にこの時期発現している転写因子Sonic Hedgehogや成長因子Fibroblast growth factor (FGF) 8などの分化誘導因子の影響により、ドーパミン、あるいは5-HT神経の前駆細胞へと分化誘導される。これらの因子を受け取った5-HT神経前駆細胞は遊走を始めることが知られており、その後、5-HT神経前駆細胞は分裂しながら正中線から外側や背側方向に遊走をはじめ、ラットではE12に初めて5-HT陽性細胞が吻側のクラスターで確認され、続いてE14には尾側のクラスターが確認される。これらの細胞はE15前後までに最終的な位置まで移動して分裂を止め、成熟ラットでは、中脳から延髄にかけて縫線核を形成する。このように縫線核の発達においてはE9-E15が重要であり、なかでもE9という時期はセロトニン前駆細胞が出現するというきわめて重要な時期で、この時期にサリドマイドやバルプロ酸などの薬剤の経胎盤の暴露が縫線核セロトニン神経系の正常な発達に対し不可逆的な悪影響を及ぼす可能性は十分考えられる。

これらの生化学的・形態学的異常に加えて、本ラットでは行動学的異常の所見も認めている。もちろんヒトとラットでは種の違いによる高次脳機能の違いは考慮しなければならない。オープンフィールドテスト、八方向放射状迷路テストなどの行動実験を繰り返し行った結果、本ラットは空間学習能力の低下に加えて、多動、そしてやみくもに動く行動、即ち非探索的行動(nonexploratory

movement) が見られた³⁾。この非探索的行動 (nonexploratory movement) はヒト自閉症にも見られる行動で興味深い²⁰⁾。

III. 厚生労働科学研究「化学物質の胎内ばく露による情動・認知行動に対する影響の評価方法に関する研究」

この流れを受けて著者らは平成 21 年度より厚生労働科学研究費補助金の補助を受け、化学物質リスク研究事業「化学物質のばく露による情動・認知行動異常に対する評価方法に関する研究」を立ち上げた。本研究班では①ヒトでの化学物質胎内ばく露の事象を動物で再現したモデル動物の解析 (研究代表者・三重大学教授・成田正明)、②胎内での化学物質ばく露の有無の判定のためのバイオマーカー確定 (発現遺伝子網羅的解析) (分担研究者・青山学院大学教授・田代朋子)、③情動・認知行動異常の評価のための非侵襲的診断法の普及 (分担研究者・文教大学教授・成田奈緒子)、④化学物質の胎内ばく露と生後の情動・認知行動の異常との関連の疫学的研究 (分担研究者・順天堂大学教授・横山和仁) に焦点をあて、化学物質の胎内ばく露によって引き起こされる生後の情動・認知行動異常を、基礎医学・臨床医学・社会医学的側面から包括的理解をめざしている²¹⁾。さらに胎生期・発達期の化学物質ばく露が危険であるとするならその種類・量・臨界期などあきらかにし、また一方、ここまでは心配ないとする種類・量・臨界期なども明らかにし、今後の厚生労働行政に資する貢献を目指し、広く国民への啓蒙を目的としている。

疫学アプローチとしては、生後の情動・認知行動異常と、体内に蓄積する有害化学物質と関連の有無を調査するため、研究協力者より生体試料として乳歯・毛髪を提供して頂き、鉛・カドミウムなどの重金属の検出の有無を試みるとともに、毛髪や乳歯などの試料提供者の詳細な発達歴を調査票で検討する全国大規模疫学調査計画を行って

おり、現在も順次解析・検討中である。これまでの調査では発達に問題のありを示唆する一定数の児童が存在することが明らかになったほか、妊娠中の異常、妊娠中の魚の摂取頻度、妊娠中の薬剤の服用歴、さらには妊娠中の居住地と幹線道路との距離などとの関連を解析中である。本疫学調査は関係諸機関の倫理委員会の承認のもと、全国都

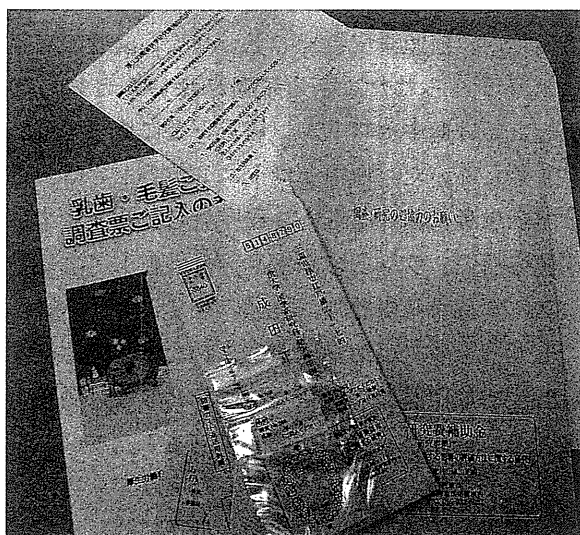


図 1. 配布する調査資料セット
説明パンフレット、質問紙、乳歯収納ビニール袋、毛髪収納ビニール袋、及び返送用封筒がセットになっている。



図 2. パンフレットの文面
研究の目的、及び個人情報を一切収集しないこと、そして採取した検体の送付方法の図解が印刷されている。

道県の教育委員会・小学校・保育園などに協力を依頼し行っている（図1, 2）。

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Plenary article

Embryonic exposure to thimerosal, an organomercury compound, causes abnormal early development of serotonergic neurons

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ABSTRACT

Even though neuronal toxicity due to organomercury compounds is well known, thimerosal, an organomercury compound, is widely used in pediatric vaccine preservation. In the present study, we examined whether embryonic exposure to thimerosal affects early development of serotonergic neurons. Thimerosal (1 mg Hg/kg) was intramuscularly administered to pregnant rats on gestational day 9 (susceptible time window for development of fetal serotonergic system), and fetal serotonergic neurons were assessed at embryonic day 15 using anti-serotonin antibodies. A dramatic increase in the number of serotonergic neurons localized to the lateral portion of the caudal raphe was observed in thimerosal group (1.9-fold increase, $p < 0.01$ compared to control). These results indicate that embryonic exposure to thimerosal affects early development of serotonergic neurons.

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Thimerosal, an organomercury compound, is known for its preservative effects on pediatric vaccines [2,12]. Thimerosal bio-transforms *in vivo* to ethylmercury and subsequently into inorganic forms of mercury [19,20], which are toxic to animals [4,7]. Therefore, accumulation of mercury through frequent vaccine administration is a concern [2,26].

The adverse effects of thimerosal have been studied extensively; neonatal administration of thimerosal induces impairment of sensitivity to pain [16] and neurodegeneration of hippocampus [17]. Although fetal organomercury poisoning (fetal Minamata disease) is known to exhibit systemic effects on fetus [5,6], little is known regarding the mechanism of action of thimerosal during the embryonic period.

Serotonergic neurons are one of the earliest neurotransmitter phenotypes to appear during the development of the nervous system [1,8,10]. In the fetal rat, serotonergic neurons were identified at around embryonic day (E) 13 (day of insemination = E1) [1,18]. However, precursor cells that were fated to serotonergic neurons are known to appear, at the latest, by E9 [25]. We previously reported that E9 is the most critical time window for early development of serotonergic neurons [15], because exposure of pregnant

rats to thalidomide resulted in caudal shift of serotonergic neurons in the dorsal raphe, suggestive of perturbed neuronal migration [13]. The effect of thalidomide was specific for the day of thalidomide administration, demonstrating that embryonic exposure at E9 is specifically crucial in the normal development of serotonergic neurons.

Since the early development of serotonergic neurons is time specific and three-dimensional [1,8,10], precise evaluation of serotonergic neuronal development by conventional immunohistochemical methods is difficult. In the present study, we utilized whole-mount preparation method for embryonic brain [1,9], which facilitates assessment of spatiotemporal data on the development of neurotransmitter system. Using this technique, we investigated whether exposure to thimerosal at E9 affects early development of serotonergic neurons.

Thimerosal administration: Pregnant Wistar rats were purchased by CLEA Japan, Inc. (Tokyo, Japan). Thimerosal (Sigma–Aldrich, St. Louis, MO) dissolved in saline (1 mg Hg/kg) was administered into pregnant rats on gestational day 9 in volume of 50 μ l, by intramuscular injection into *glutei maximi*. For control group, saline was administered in the same way. Three dams for each group (thimerosal vs control) were examined. All animal experiments were authorized by the Animal Research Committee in Mie University.

Flat whole-mount preparation of rat brain: The procedure for preparing flat whole-mount hindbrain has been described previously [1,9,21,22]. In brief, E15 fetuses were dissected out and the

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embryonic brains were cut along the dorsal midline. The brain was then opened and whole mounted flatly with the ventricular side down.

Serotonin immunostaining: Flat whole-mounted brain was fixed with 4% paraformaldehyde for overnight at 4 °C. The immunostaining procedures follow as Muguruma and Watanabe [14] and Watanabe et al. [24]. In brief, the preparations were incubated in Tris-buffered saline, pH 7.5 (TBS), containing 2% Triton X-100 (TBST), then incubated in methanol containing 0.3% hydrogen peroxide. Subsequently, they were incubated in 10% normal goat serum (NGS) and then incubated in anti-serotonin antibody (Invitrogen, Camarillo, CA, 1:200 dilutions) as a primary antibody in 2% NGS-TBST for overnight at room temperature. The preparations were rinsed with TBST, followed biotinylated secondary anti-rabbit IgG (Chemicon, Temecula, CA, 1:200 dilution) and Vectastain ABC Elite kit (Vector laboratories, Burlingame, CA). To develop the staining, tissue was incubated in 0.05% 3,3'-diaminobenzidine tetrahydrochloride in TBS with 0.01% hydrogen peroxide. Tissues were gently coverslipped, observed under a light microscope and photographed with digital camera DXM1200F (Nikon, Tokyo, Japan). The numbers of serotonin-immunopositive cells in the lateral portion of the caudal raphe (see results) were counted. The cells were counted blindly to exclude the possibility of the

bias. Consistent results were obtained from three independent experiments.

To evaluate the early development of serotonergic neurons, whole-mount preparation method for the fetal rat brain was used [1,9], which facilitates morphological examination at the early embryonic stage of serotonergic neurons developing spatiotemporally in the fetal brain. Fig. 1A shows the two major known clusters in the hindbrain of the E15 rat embryo, with rostral and caudal clusters of raphe nuclei [10]. Fig. 1A also shows descending fibers from the caudal raphe.

Differences in the basic structure of the raphe between the thimerosal and control groups were not noted initially. However, following careful observation, we discovered a difference in the smaller clusters of serotonergic neurons in the lateral portion of the caudal embryonic hindbrain, i.e., in the dorsal area of the major caudal raphe nucleus. The clusters of control embryos were shown in boxed area in Fig. 1A. The cells observed were bipolar, spindle-shaped, and were oriented at 90° to the floor plate (Fig. 1B and C), suggesting development and migration of serotonergic neurons.

To evaluate whether fetal E9 administration of thimerosal affects the early development of the serotonergic system, neurons in the lateral portion of the caudal raphe were counted. Representative photographs were shown in Fig. 2A. The counting area was

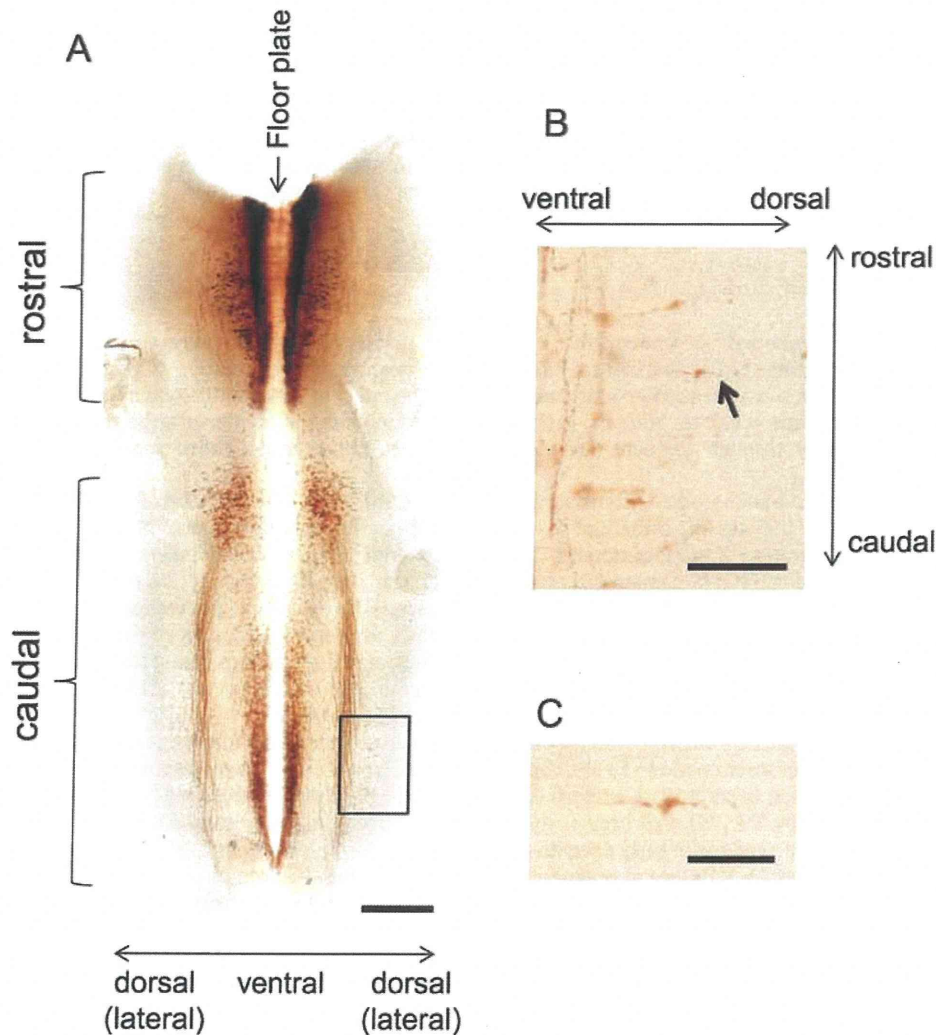


Fig. 1. Serotonin immunostaining of an E15 flat whole-mount preparation. (A) Low-magnification view of a flat whole-mount preparation. (B) High-magnification view in the lateral portion of the caudal raphe (boxed area in A). The serotonergic neurons were bipolar spindle-shaped along a ventral–dorsal axis (arrow). (C) An enlargement of the serotonergic neuron arrowed in B. Scale bar: 500 μm (A), 100 μm (B), and 50 μm (C).

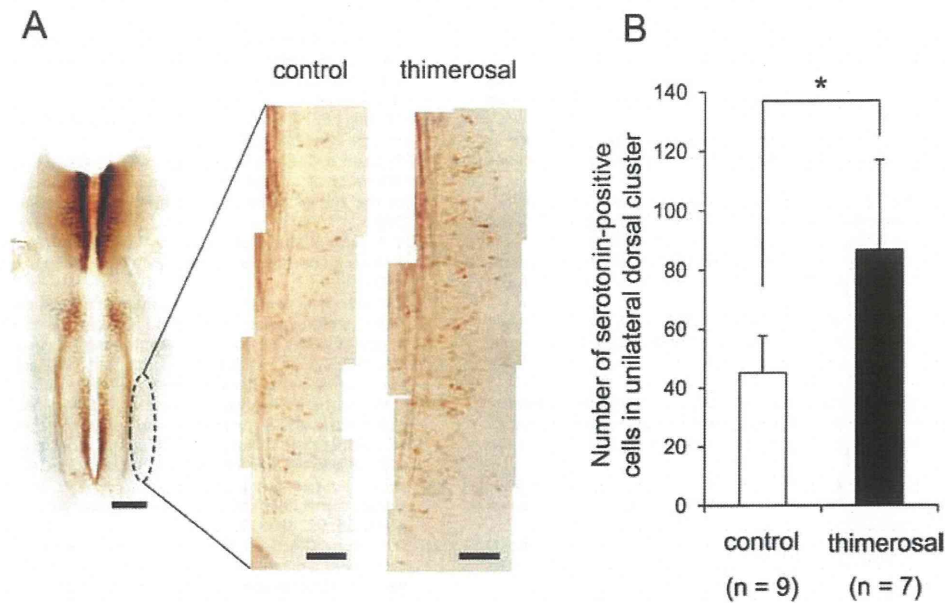


Fig. 2. Effect of thimerosal of serotonergic neuron in the E15 rat whole-mount preparation. (A) Representative photographs of the cluster of serotonergic neurons in the lateral portion of the caudal raphe in control and thimerosal injection. Low-magnification view is also shown (left panel). (B) Comparison of serotonin-immunopositive cells in lateral portion of the caudal cluster between control and thimerosal injection. Numbers represent the average of each determination \pm S.D. from one of three independent experiments. Scale bar: 500 μ m (left in A), 100 μ m (control and thimerosal). * $p < 0.01$ (Student's unpaired *t*-test).

encircled with dashed line. A dramatic increase in lateral spread of these neurons was observed in the thimerosal group ($n = 7$) when compared with the control group ($n = 9$) (Fig. 2B, Student's unpaired *t*-test, $p < 0.01$). These results indicate that exposure to thimerosal at E9 affects early serotonergic development.

In the present study, using the whole-mount preparation method, we demonstrated a dramatic increase in the number of serotonergic neurons localized in the lateral portion of the caudal raphe after exposure to thimerosal at E9. This finding indicates that thimerosal, an organomercury compound, affects the embryonic development of serotonergic neurons.

Embryonic exposure to thimerosal affected early serotonergic neurons in the lateral portion of caudal raphe. Although the precise roles of these neuronal clusters need to be elucidated, similar clusters, which expressed serotonergic neurons during early development, were reported [1,9]. Individual neurons of this cluster were observed to be orthogonally oriented to the floor plate, suggesting lateral migration of developing serotonergic neurons. Thus, they are considered to be important markers in the evaluation of serotonergic development. Therefore, an increase in cell number spreading laterally within the caudal cluster of the raphe strongly suggests that E9 exposure to thimerosal affects early development of serotonergic system.

Initial microscopic inspections at E15 after exposure to thimerosal at E9 did not seem to reveal any alteration in the general structure of the rostral and caudal raphe. So the results of this study are limited to morphologic change in "minor" population of serotonergic neurons.

Nevertheless, our results are of significance in that we found for the first time that embryonic exposure to mercury affects morphological changes in the developing serotonergic system. Although the embryonic toxicity of mercury is well known in fetal Minamata disease [5,6], its relation to serotonergic development has not been established yet. The present study may offer evidence for elucidation of mercury toxicity to serotonergic system.

Thimerosal has been widely used as a preservative in pediatric vaccines [2], although it was gradually eliminated from vaccines in USA [3] and EU [23]. Our present data do not necessarily

prove any direct and/or irreversible adverse effects of embryonic exposure to thimerosal. However, as Lawton et al. suggested, thimerosal inhibits the extension of neural processes in neuroblastoma (Neuro2a), thus implying an effect on neural development [11].

Future studies aimed at investigating the adverse effects of thimerosal on embryonic development, and the functions of minor serotonin clusters, *i.e.*, the lateral spread of neurons in the caudal cluster, are required.

In summary, it can be concluded that embryonic exposure to thimerosal increased the number of serotonergic neurons localized in the lateral portion of the caudal raphe. This result suggests that thimerosal affects the embryonic development of serotonergic neurons.

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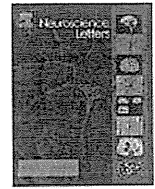
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Commentary

Animal models of the human mind: Is there anything like being autistic?

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"Even if one agreed that the scientific agenda outlined here may be adequate to handle the issues of awareness and volition, there is another aspect of consciousness that needs to be confronted and that is the nature of subjectivity (. . .) Are the purple you see and the jasmine you smell identical to the purple that we see and the jasmine that we smell? The fact that conscious experience is uniquely personal and intensely subjective raises the question whether it is ever possible to determine objectively some common characteristics of experience." [2]

"We can also ask with respect to an experience what it was like to undergo it, and the answer, if there is one, will specify whether one found it enjoyable or unpleasant, interesting or boring, frightening or exciting, etc. None of this is mysterious, surprising or baffling. Nor is it the key to unlocking the mysteries of consciousness. For there are no mysteries—only empirical ignorance and conceptual mystification. Disentangling one of the roots of the conceptual confusions that conjure qualia into being is a first step towards the demystification of consciousness." [8]

With the exception of several remarkable genetic variants identified in a minority of patients, the manifestations of autism appear to be weakly or inconclusively associated with large series of genomic abnormalities [1], while many autistic individuals exhibit none of them. Thus, the question of causal or contributory environmental influences remains at the fore. The quest for extra-genetic autism risk factors (whether toxic, vaccinal or medicinal) continues to be vigorous. Curiously, all causal models contemplated are deterministic rather than risk-estimating in the proper sense of the term, such that it is commonly hypothesized that the sum of one or more genetic abnormalities and one or more co-causal environmental agents must lead to the disorder. But such reductionism is necessary only provisionally to approach experimental problems more easily and will undoubtedly be superseded by proper risk

(and uncertainty) estimates if these concerted causal approaches bear results.

In contrast with the descriptive task of human gene identification, experimental environmental research is, by nature, mechanistic. Epidemiologic observations in man have led to the formulation of toxicological hypotheses amenable to testing. However, the strength of these hypotheses is proportional to the robustness of their epidemiological correlate, as it can be expected that increasingly strong causative action (i.e., toxic agent dose) will necessarily lead to more notable consequences (e.g., severity of observable abnormal behavior). Aside from the phenomenon of the phenotypic heterogeneity of autism or, more problematically, from the more mundane obstacle of uneven diagnostic acumen, environmentally focused autism research has yielded limited or negative epidemiological evidence, including, among other leads, that related to immunizations [12,13]. However, epidemiology tends to blur the individual, subordinate the subset to the larger population sample and never account for "outliers", who defy explanation. Therefore, it seems legitimate to ask the question of whether, in the context of a predisposing (however unique, rare or still undefined) genetic background, toxicity does bring about autism.

Studies by Ida-Eto et al. [9] demonstrate that exposure of the rat embryo to thimerosal perturbs the early development of fetal serotonergic neurons. By studying E15 fetal rat hind-brains in flat whole-mount preparation following administration of thimerosal on gestational day 9 via intramuscular injection, the authors discovered an increase in the number of serotonergic neurons located in small clusters at the lateral portions of the caudal raphe nucleus in thimerosal-exposed embryos. Thimerosal, a much-debated organomercury preservative, was generally used for the prevention of contamination of vials containing vaccines for use in multiple inoculations after opening. Since 2001, all vaccines manufactured for the U.S. market and routinely recommended for children ≤ 6 years of age have contained no thimerosal or only trace amounts (≤ 1 microgram of mercury per dose remaining from the manufacturing process), with the exception of inactivated influenza vaccine. In addition, all of the routinely recommended vaccines that had been previously manufactured with thimerosal as a preservative (some formulations of DTaP, *Haemophilus influenzae* b conjugate (Hib), and hepatitis B vaccines) expired before January

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