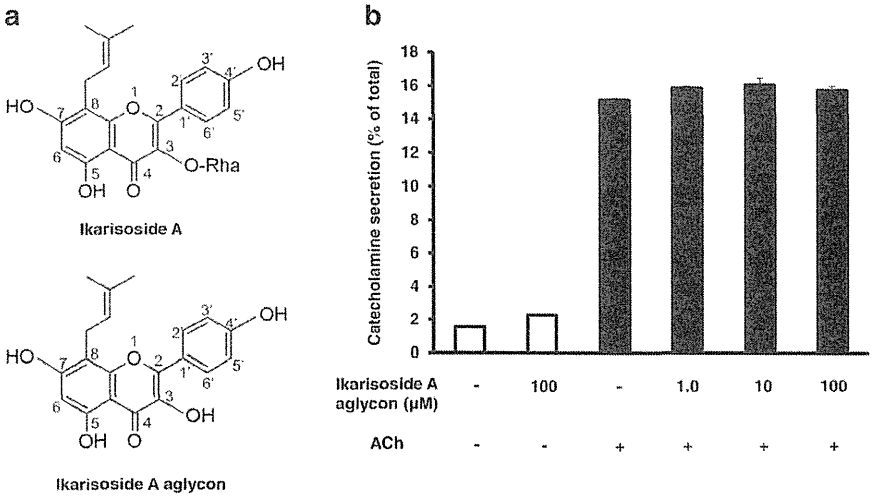

Fig. 6 Structure of ikarisoside A and its aglycon (**a**) and effect of aglycon of ikarisoside A on ACh-induced catecholamine secretion (**b**). **a** Structure of ikarisoside A and its aglycon (3,5,7-trihydroxy-2-(4-hydroxyphenyl)-8-(3-methylbut-2-enyl)-4*H*-chromen-4-one). **b** After preincubation with cells with or without aglycon of ikarisoside A (1–100 μM) for 10 min, the cells (10^6/well) were incubated with or without aglycon of ikarisoside A (1–100 μM) and ACh (300 μM) for another 10 min at 37 °C. Catecholamines secreted into the medium were expressed as a percentage of the total catecholamines in the cells. Data are means ± SEM from three separate experiments carried out in triplicate

to that of catecholamine secretion. In the exocytotic secretion of catecholamines, Ca^{2+} plays an indispensable role as the coupler in the stimulus-secretion coupling (Douglas and Rubin 1961, 1963). From these findings, it is likely that ikarisoside A inhibits ACh-induced catecholamine secretion by suppressing nAChR-ion channels. We investigated the inhibitory mode of ikarisoside A on nAChR-ion channels. Even when the concentration of ACh was increased, the inhibitory

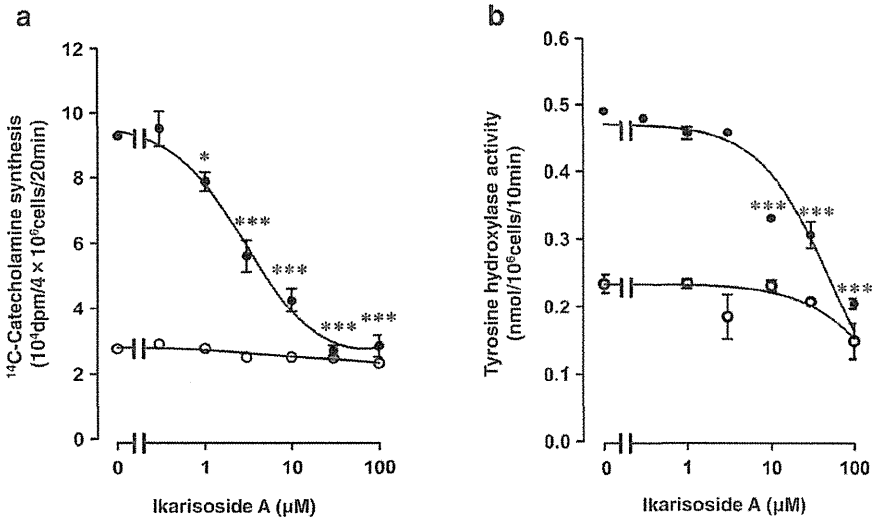

Fig. 7 Effects of ikarisoside A on ^{14}C-catecholamine synthesis from [^{14}C]tyrosine (**a**) and tyrosine hydroxylase activity (**b**) in the cells. **a** After preincubation for 10 min with or without ikarisoside A (0.1–100 μM), cells (4 × 10^6/dish) were incubated with L-[U-^{14}C] tyrosine (20 μM, 1 μCi) in the presence or absence of ikarisoside A (0.1–100 μM) and with (*black circle*) or without (*white circle*) 300 μM ACh at 37 °C for 20 min. The ^{14}C-catecholamines formed were measured. **b** After preincubation with or without ikarisoside A (0.1–100 μM) for 10 min, cells (10^6/well) were incubated with L-[1-^{14}C] tyrosine (18 μM, 0.2 μCi) in the presence or absence of ikarisoside A (0.1–100 μM) and with (*black circle*) or without (*white circle*) 300 μM ACh at 37 °C for 10 min, and tyrosine hydroxylase activity was measured. Data are means + SEM from three separate experiments carried out in triplicate. $^*P < 0.05$ and $^{***}P < 0.001$, compared with ACh alone (analyzed by one-way ANOVA with Dunnett's multiple comparison post hoc test)

effect of ikarisoside A on ACh-induced secretion of catecholamines was not overcome, suggesting a noncompetitive inhibition and that ikarisoside A acts at a site different from that for ACh binding. A previous review (Lena and Changeux 1993) reported that the site at which noncompetitive blockers act lies at the interface between the nicotinic receptor protein and the membrane lipids.

In the *Xenopus* oocytes expressed with α3β4 nAChRs, ikarisoside A directly inhibited ACh-induced current. The IC_{50} values of ikarisoside A for $^{22}Na^+$ influx in adrenal medullary cells and for Na^+ current in the oocytes were 2.96 and 0.48 μM, respectively. The IC_{50} in the bovine adrenal medullary cells is 6.2-fold bigger than that of the drug in the oocyte system. Although the reason for the discrepancy of the IC_{50} between the two systems is not yet clear, the discrepancy may be explained in the following way. (1) A maximally effective concentration of ACh was used for the $^{22}Na^+$ influx experiments in bovine adrenal medullary cells but the half-maximal concentration was used for the Na^+ current in the oocyte system. (2) In the oocyte expression system, there may be some changes in the test compound potency compared to that of the method using mammalian cells, i.e., a decrease (Lambert et al. 2001; Akk et al. 2008) or an increase (Pintado et al. 2000) in the sensitivity of test compounds. (3) Bovine adrenal medullary cells express multiple nAChR subtypes such as α3β4 (Criado et al. 1992; Garcia-Guzman et al. 1995), α3β4α5 (Campos-Caro et al. 1997), and α7 (Lopez et al. 1998). We should study above possibilities and examine the effect of ikarisoside A on the function of nAChRs in other mammalian cells.

Structure-activity relationship of ikarisoside A for inhibition of nAChR-ion channels

In the present study, we used four flavonol glycosides derived from the *Epimedium* species. Ikarisoside A, but not the other three flavonols, inhibited the functioning of nAChR-ion channels. Judging from the differences in their structures, ikarisoside A has a hydroxyl group at the 7 position in the structure whereas other three have a glucose moiety at this position, suggesting that a glucose moiety at the 7 position may induce stereo-specific interference when flavonol glycosides interact with nAChRs. Furthermore, the inhibition of ACh-induced secretion by ikarisoside A disappeared by the removal of the rhamnose moiety at the 3 position from ikarisoside A. These findings suggest that the rhamnose moiety at the 3 position of ikarisoside A is essential to inhibit the function of nAChR-ion channels.

Inhibitory effect of ikarisoside A on catecholamine synthesis

Ikarisoside A inhibited not only catecholamine secretion but also reduced catecholamine synthesis in ACh-stimulated cells. In the regulation of catecholamine synthesis, Ca^{2+} plays an important role as the coupler in the stimulus-synthesis coupling (Yanagihara et al. 1987) as well as in the stimulus-secretion coupling (Douglas and Rubin 1961, 1963). In the present study, we observed that ikarisoside A suppressed the $^{22}Na^+$ influx and the subsequent $^{45}Ca^{2+}$ influx by inhibiting nAChR-ion channels. Therefore, it is likely that ikarisoside A inhibits catecholamine synthesis and tyrosine hydroxylase activity induced by ACh via the suppression of Ca^{2+} influx in cultured bovine adrenal medulla cells. In harmony with this view, the IC_{50} values of ikarisoside A for inhibition of $^{22}Na^+$ and $^{45}Ca^{2+}$ influx and for inhibition of catecholamine synthesis and tyrosine hydroxylase are very similar.

Pharmacological significance of the inhibitory effects of ikarisoside A on adrenal medullary functions

The human serum concentration of ikarisoside A has not been reported yet. Several previous in vitro studies reported that ikarisoside A at 5.0–20 μM inhibits osteoclastogenic differentiation and nitric oxide synthase in murine monocyte/macrophage cell line RAW264.7 cells (Choi et al. 2008, 2010) and induces neurite outgrowth activity in PC12h cells at 10 μM (Kuroda et al. 2000). In the present study, we observed a significant inhibition of ikarisoside A at 0.1 and 1.0 μM in ACh-induced current in *Xenopus* oocytes and ACh-induced synthesis and secretion of catecholamines, respectively.

It is well known that adrenaline and noradrenaline have an important role in the regulation of normal function in the central and peripheral sympathetic nervous systems. Under strong and prolonged stress, an increased catecholamine release may occur, which possibly induces cardiovascular diseases such as hypertension, atherosclerosis, coronary heart disease, and heart failure (Yanagihara et al. 2014). Chronic heart failure is reported to be associated with the activation of the sympathetic nervous system as manifested by increased circulating catecholamines (Westfall and Westfall 2011). Furthermore, Hara et al. (2011) reported that the stress hormone adrenaline stimulates β_2-adrenoceptors, which activates the Gs protein/cyclic AMP-dependent protein kinase and the β-arrestin-mediated signaling pathway, reduces the p53 level, and induces DNA damage.

Our previous studies reported that daidzein, a soy isoflavone, (Liu et al. 2007) and nobiletin, a citrus polymethoxy flavone, (Zhang et al. 2010) suppress the secretion and synthesis of catecholamines induced by ACh in cultured bovine adrenal medullary cells. In addition to these flavonoids, ikarisoside A also may protect the hyperactive catecholamine system induced by strong stress or emotional excitation which evokes the secretion of ACh from the splanchnic nerves. Further in vivo experiments will provide more conclusive information on ikarisoside A and promote the development of a

therapeutic drug for stress-induced disorders associated with mental or cardiovascular diseases.

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Conflict of interest The authors declare that they have no competing interests.

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Ⅴ. 化学物質リスク研究事業・班会議資料

平成 27 年 9 月 12 日開催

平成27年度厚生労働科学研究費補助金

化学物質リスク研究事業

「個体の成長期における毒性メカニズムに基づく新規in vitro発達神経毒性評価法に関する研究」

班会議　議事録

日時:平成 27 年 9 月 12 日(土)　15 時 00 分～19 時 00 分

場所:ソニックシティビル　7階　703会議室

(〒330-8669　埼玉県さいたま市大宮区桜木町1-7-5)

出席者: 上野　晋、笛田由紀子(産業医大)、吉田祥子(豊橋技術科学大)、諫田泰成(国衛研)

※関野祐子(国衛研):当会議に出席できないため事前打合せを行い、議事内容について了承済み。

(以上、敬称略、順不同)

議事:

1. 今年度に評価する化学物質候補の選定、およびその予備評価のための研究計画に関する討議

(15:00～16:25)

(休憩)

2. in vivo 評価系の結果のまとめと学術論文作成に向けた発表内容の構成に関する討議

(16:35～19:00)

以上

平成27年度班会議

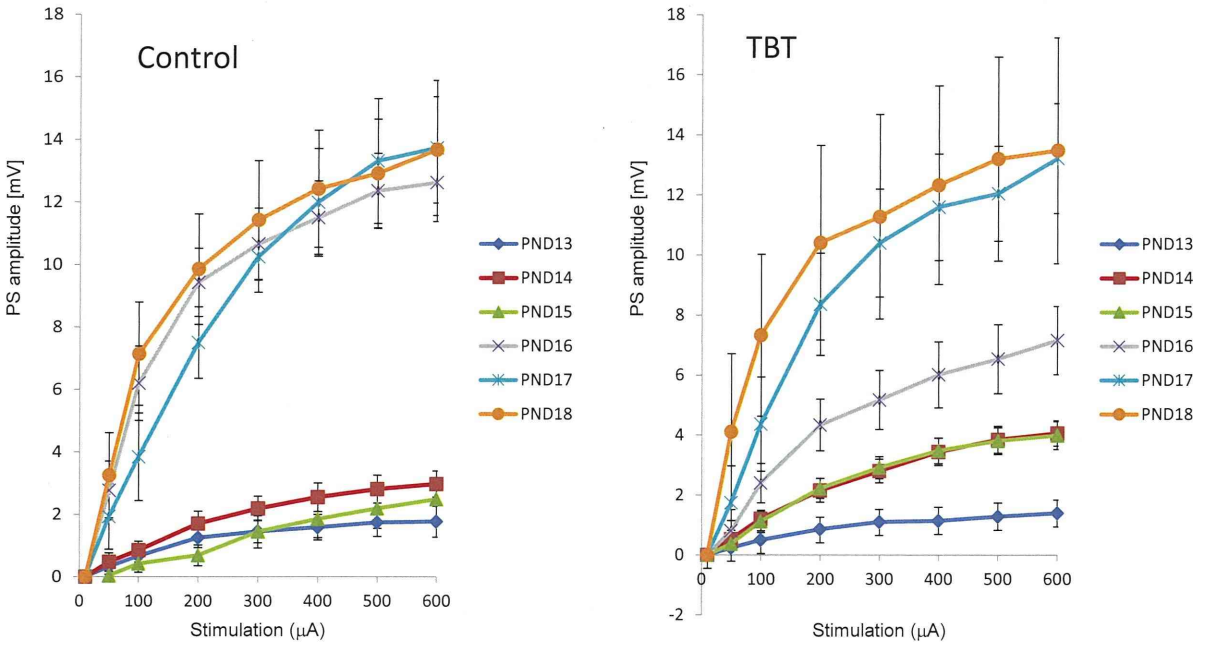
TBT実験の進捗状況

平成27年5月23日（土）
国立医薬品食品衛生研究所にて
産業医科大学　上野晋、笛田由紀子

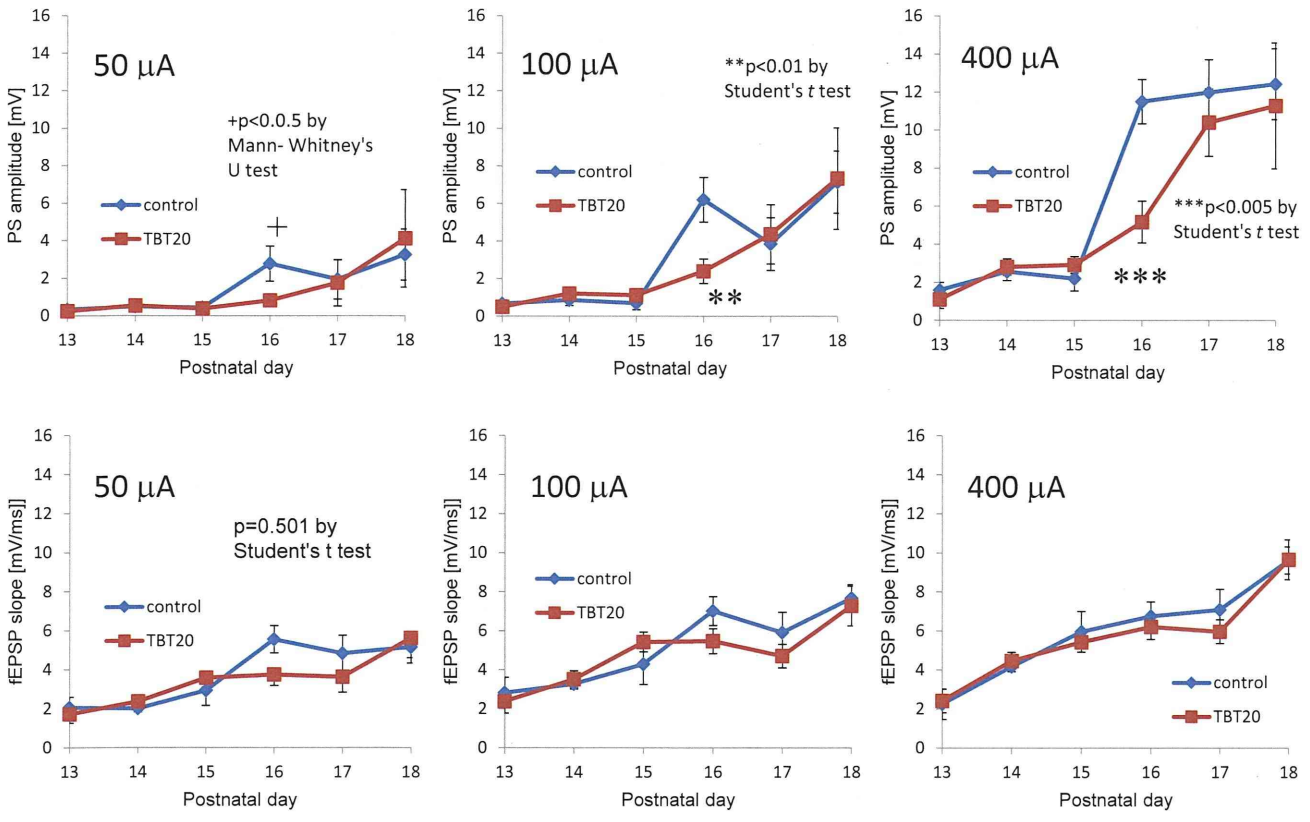
方法

- PEGを蒸留水で2倍に希釈して、20mg/kgとなるようにTBT溶液を加えた。
- Day15の午前11時～午後2時の間に、軽い麻酔下（女性がラットを保持できる）でTBT/希釈PEGを経口投与。対照群には希釈PEGのみ投与。
- PND1（出産日翌日）に体重の重い順に産仔数を10匹にした（♂優先）。仔の数が10匹に満たない場合は、体重を測定してそのまま授乳させた。
- 本日の班会議までに解析した腹数は、対照群5腹、TBT投与群6腹
- 体重増加の抑制がかかった仔が、対照群に1匹、投与群に3匹（うち1匹死亡）いた⇒TBTの影響かどうか、腹数を増やして要観察。
- PND13-18で海馬スライスを作製し、解析。
- VPA投与実験の時のように、刺激応答曲線、発達曲線を作製し、対照群と投与群で比較した。

対照群とTBT群の刺激応答曲線



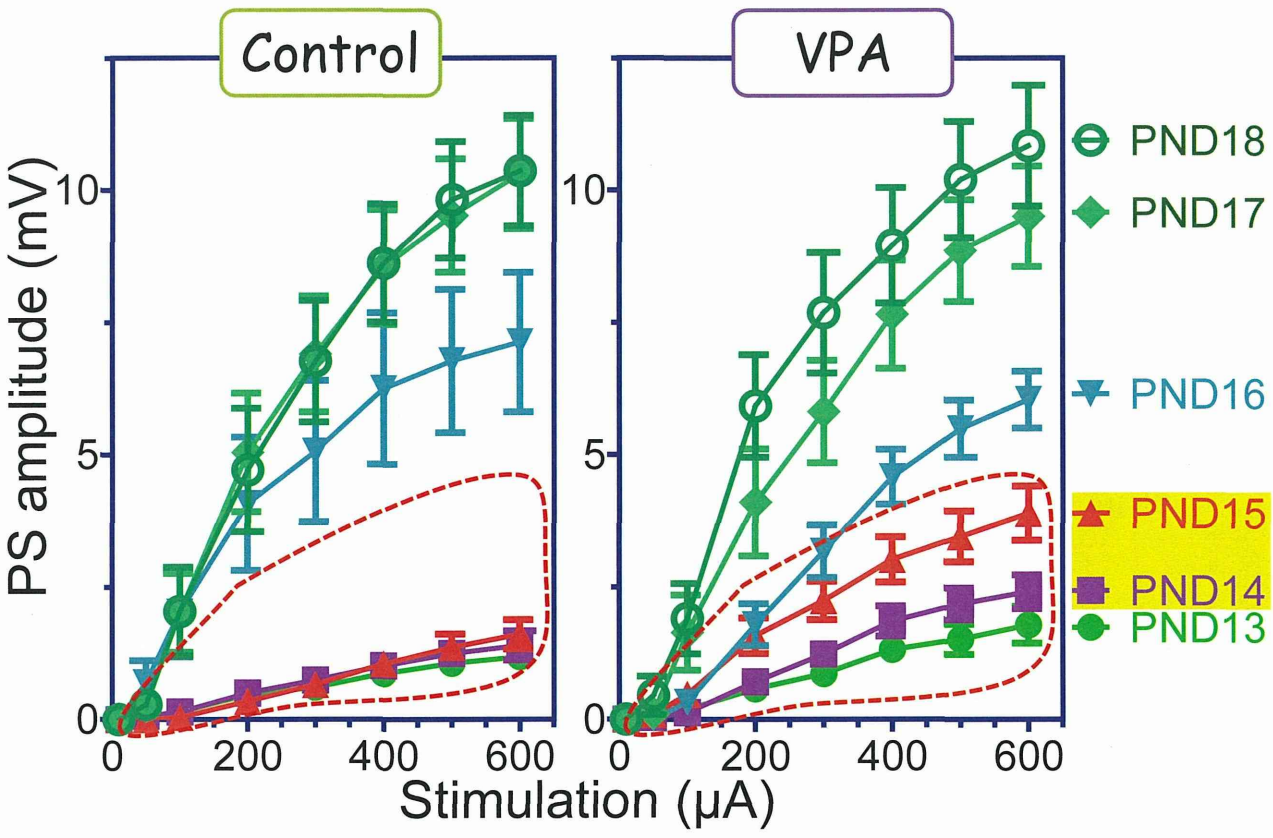
TBT投与群の発達曲線



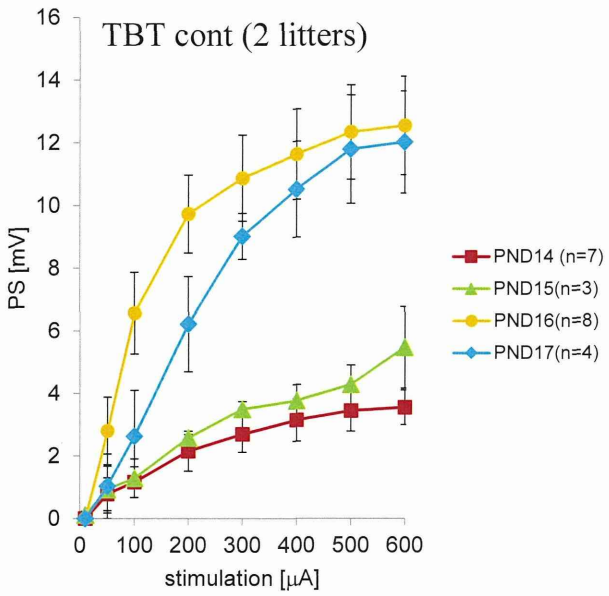
刺激応答性について
PSの結果

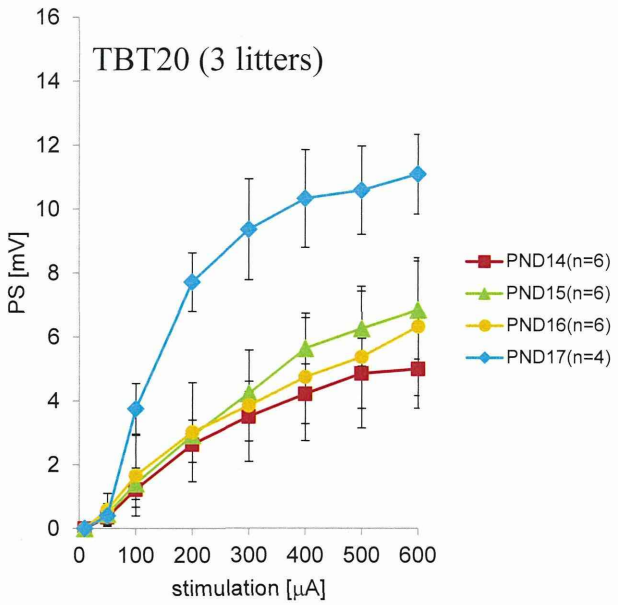
- VPA
- TBT
- 1-BP

VPA胎生期曝露により生後14－15日齢での刺激応答性の発達が亢進する

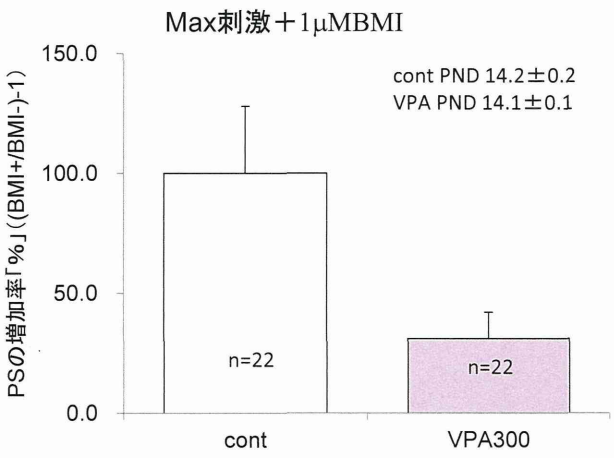


TBT:
刺激応答曲線

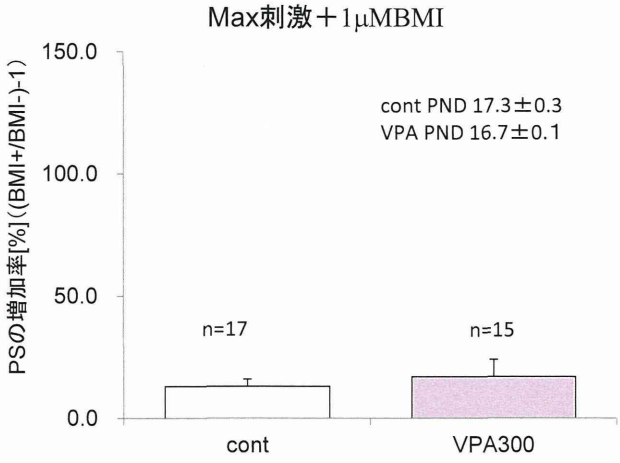




single responseへのBMI(1μM)の影響



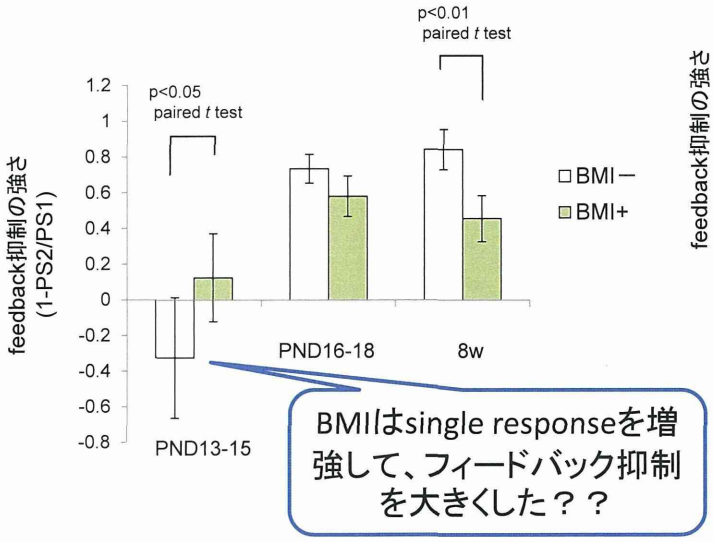
PND13-15



PND16-18

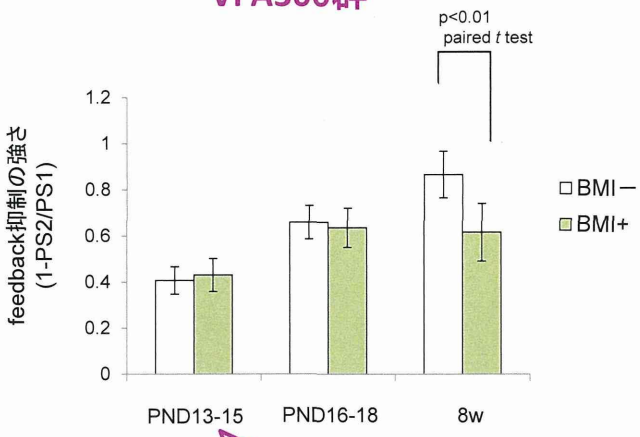
フィードバック抑制の強さへのBMI(1μM)の影響

feedback抑制の発達に伴うBMIの効果: 対照群



BMIはsingle responseを増強して、フィードバック抑制を大きくした？？

feedback抑制の発達に伴うBMIの効果: VPA300群



BMIはsingle responseに全く影響しない？