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理事長 中島 章 殿

研究室で撮影した本人のスナップ写真、及び発表論文のコピーを添付

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研究テーマ 隔離飼育ストレス誘発のペントバルビタール睡眠短縮におけるDiazepam Binding Inhibitor(DBI)の役割

2．本年度の研究業績

(1) 学会・研究会等においての口頭発表 (有) ・ 無 (学会名・内容)

第49回日本薬理学会北部会

In volvement of mitochondrial benzodiazepine receptor in social isolation-induced decrease in pentobarbital sleep in mice (口演)

(2) 学会誌等に発表した論文 (有) ・ 無 (雑誌名・論文名)

1. *Life Sciences* ~~(in press)~~ vol. 64 p1779-1784, 1999
   Possible involvement of diazepam binding inhibitor and its fragment octadecaneuropeptide in social isolation stress-induced decrease in pentobarbital sleep in mice
   著者：董 而 博、松本欣三、渡邊裕司

2. *Life Sciences* (in press)
   Involvement of peripheral type of benzodiazepine receptor in social isolation stress-induced decrease in pentobarbital sleep in mice
   著者：董 而 博、松本欣三、渡邊裕司

3. *Neuroscience Research* (in press)
   Diazepam binding inhibitor (DBI) gene expression in the brains of socially isolated and group-housed mice
   著者：董 而 博、松本欣三、東田道久、金子喜彦、渡邊裕司

3．今後の研究計画

これまでに脳内 GABA-A 受容体機能を制御する脳内物質の動態がストレス病態発見と密接に関連する可能性を検討していた．今後，脳内 GABA-A 受容体機能を制御する内因性物質候補の動態と薬物誘発睡眠時間の短縮や攻撃性などのストレス病態発見との関連性を定量的に解析する．この研究より，内因性物質の生理的病理学的な役割を突き止められることが期待され，またヒトにおける慢性的心理的ストレスに起因する疾患の予防，治療法の確立が可能となる．更に　内因性物質の活性を調節する和漢薬，伝統薬物，合成医薬品を探索することによって新しい機序の抗ストレス薬の開発の突破口となるものと期待される．

4．研究指導者の意見

本研究は、1）長期隔離飼育ストレスにより内因性リガンドである DBI が中枢性および末梢性の BDZ 受容体に結合し、直接的および間接的に $GABA_A$受容体機能を低下させ、マウスの PB 睡眠を縮短する、2）隔離ストレスは視床下部 DBI 遺伝子レベルの減少をもたらす、ことを明らかにした。
このような隔離飼育ストレスで惹起せれる中枢機能変化の研究が、ストレスに起因した精神神経疾患の病因解明や治療薬の開発に有用であることを示した点が評価された。

研究指導者氏名　渡辺裕司　渡印

5．研究報告

別紙形式を参考に、報告本文4000字以上で報告して下さい（枚数自由・ワープロ使用）

タイトル・要旨等は日本語で、KEY WORDS以下は日本語或いは英語で記入して下さい。

研究成果の発表予定がある場合は発表原稿・抄録集等を添付して下さい。

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隔離飼育ストレス誘発のペントバルビタール睡眠短縮における Diazepam Binding Inhibitor (DBI)の役割

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本研究はストレスに起因する不眠症、うつ病、不安神経症などの発症原因の解明やそれらの疾患の治療薬の開発に資することを目的として、長期隔離飼育マウスの情動ストレスモデルとしての有用性を検討したものである。

１)長期隔離飼育マウスではペントバルビタール(PB)誘発睡眠は短縮されるので、この PB 誘発睡眠を指標として、diazepam binding inhibitor (DBI) や中枢型のベンゾジアゼピン（BZD）受容体拮抗薬 flumazenil の作用を検討したが、影響は認められなかった。しかし、 flumazenil を脳室内投与すると隔離群の PB 誘発睡眠のみが延長され、DBI 前処置により抑制された。他方、DBI は用量依存的に群居群マウスの PB 誘発睡眠を短縮し、flumazenil の前処置によりその結果は遮断された。

これらの結果は、長期の隔離飼育によりマウス脳内における DBI の量または活性の変化が生じ、$GABA_A$受容体複合体上の中枢性 BZD 受容体(CBR)を介する$GABA_A$受容体機能低下し、マウスの PB 催眠活性の減少に寄与している可能性を示唆している。

２）グリア細胞のミトコンドリア膜上に存在する末梢性 BZD 受容体(PBR)は、ニューロステロイド産生を調節し、$GABA_A$受容体機能に対して間接的に働く事が知られている。 $GABA_A$受容体の非競合的拮抗薬である Pregnenolone sulfate を脳室内投与すると隔離群の PB 睡眠は影響されず、群居群で用量依存的な短縮が起こった。一方、末梢性 BZD 受容体作動薬 FGIN-1-27 や同拮抗薬 PK11195 や pregnenolone（神経ステロイドの precursor）は逆に隔離群の PB 誘発睡眠のみを用量依存的に延長した。更に隔離群における FGIN-1-27 や PK11195 や Pregnenolone の効果は単独では作用しない Pregnenolone sulfate により拮抗された。

従って、隔離群の脳内では（１）$GABA_A$受容体作動性神経ステロイドの活性低下、（２）$GABA_A$受容体作動性神経ステロイドの活性上昇、あるいは（３）（１）と（２）の両方の変化が生じて $GABA_A$受容体機能低下し、PB 誘発睡眠が短縮される可能性が推察された。

（３）脳内の DBI mRNA 分布を in situ hybridization 法で測定した結果、 DBI 遺伝子は視床下部や小脳に分布することが明らかとなった。脳内の DBI mRNA 量を RT-PCR 法で測定した結果、視床下部の DBI 遺伝子量が隔離飼育期間の長さに依存して減少することが明らかとなった。この結果は DBI 遺伝子が隔離マウスにおいて過剰発現するという仮説に反するが、 DBI ペプチドによるネガティ

ブフィードバック、 DBI よりも強い活性を持つ他の内因性基質、ストレス関与ホルモン等による影響のためであると考えられた。視床下部-下垂体-副腎皮質系は種々のストレス刺激に応じて即座に活性化され、糖質コルチコイドの分泌量を増加させるので、糖質コルチコイドが脳内における DBI 遺伝子発現に影響を及ぼす因子の一つである可能性がある。

Key words: 隔離飼育ストレス(Social isolation stress), ベンゾジアゼピン受容体(Benzodiazepine (BZD) receptor), $GABA_A$受容体($GABA_A$ receptor), Diazepam binding inhibitor (DBI), mRNA, *In situ* hybridization, ペントバルビタール誘発睡眠 Pentobarbitol-induced sleep.

研究報告

Studies on the Involvement of Diazepam Binding Inhibitor (DBI) in Social Isolation Stress-induced Decrease in Pentobarbital Sleep in Mice

Social isolation stress causes a decrease in the hypnotic action of pentobarbital (PB) in mice. Investigations have revealed that the alteration in PB sleep by the stress is due to dysfunction of GABAergic systems in the brain, and that endogenous substance(s) with an inverse benzodiazepine (BZD) receptor agonist property play(s) a role in this alteration. Diazepam binding inhibitor (DBI), a polypeptide isolated from mammalian brains, is a putative endogenous BZD receptor ligand since it has a high affinity to BZD receptors, negatively modulates $GABA_A$ receptor ($GABA_A$-R) function and produces anxiety, aggression, etc. The purpose of this study is to evaluate the possible involvement of DBI and octadecaneuropeptide (ODN), a peptide spliced from DBI, in social isolation-induced decrease in the hypnotic action of PB.

Section I. Possible Involvement of DBI and Its Fragment ODN in Social Isolation Stress-induced Decrease in PB Sleep in Mice [1)]

To test the possible participation of DBI and ODN in isolation stress-induced reduction of PB hypnotic activity in mice, male ddY mice (18-20 g, 4 weeks old) were either housed in groups or isolated individually for 6 weeks before experiments. PB-induced sleep was measured as the duration between the loss of the righting reflex and its return following intraperitoneal (i.p.) injection of PB-Na (50 mg/kg). The test drugs were given intracerebroventricularly (i.c.v.) 30 min before PB. DBI and ODN (3 and 10 nmol) dose-dependently decreased PB-induced sleep in group-housed but not isolated mice. In contrast, flumazenil (16.5-33 nmol), a BZD receptor antagonist, reversed the PB sleep in isolated mice to the normal level without affecting that in group-housed animals. The effects of DBI and ODN in group-housed mice were significantly blocked by flumazenil (33 nmol). In contrast, these peptides (10 nmol) significantly attenuated the reversing effect of flumazenil in isolated mice. These results suggest that the change in the activity or content of DBI and ODN in the brain occurs following social isolation and contributes partly to the decrease in the hypnotic action of PB in mice partly by down-regulating

$GABA_A$-R function via the central type BZD receptor (CBR) on the $GABA_A$-R complex (see Fig.1 and 2).

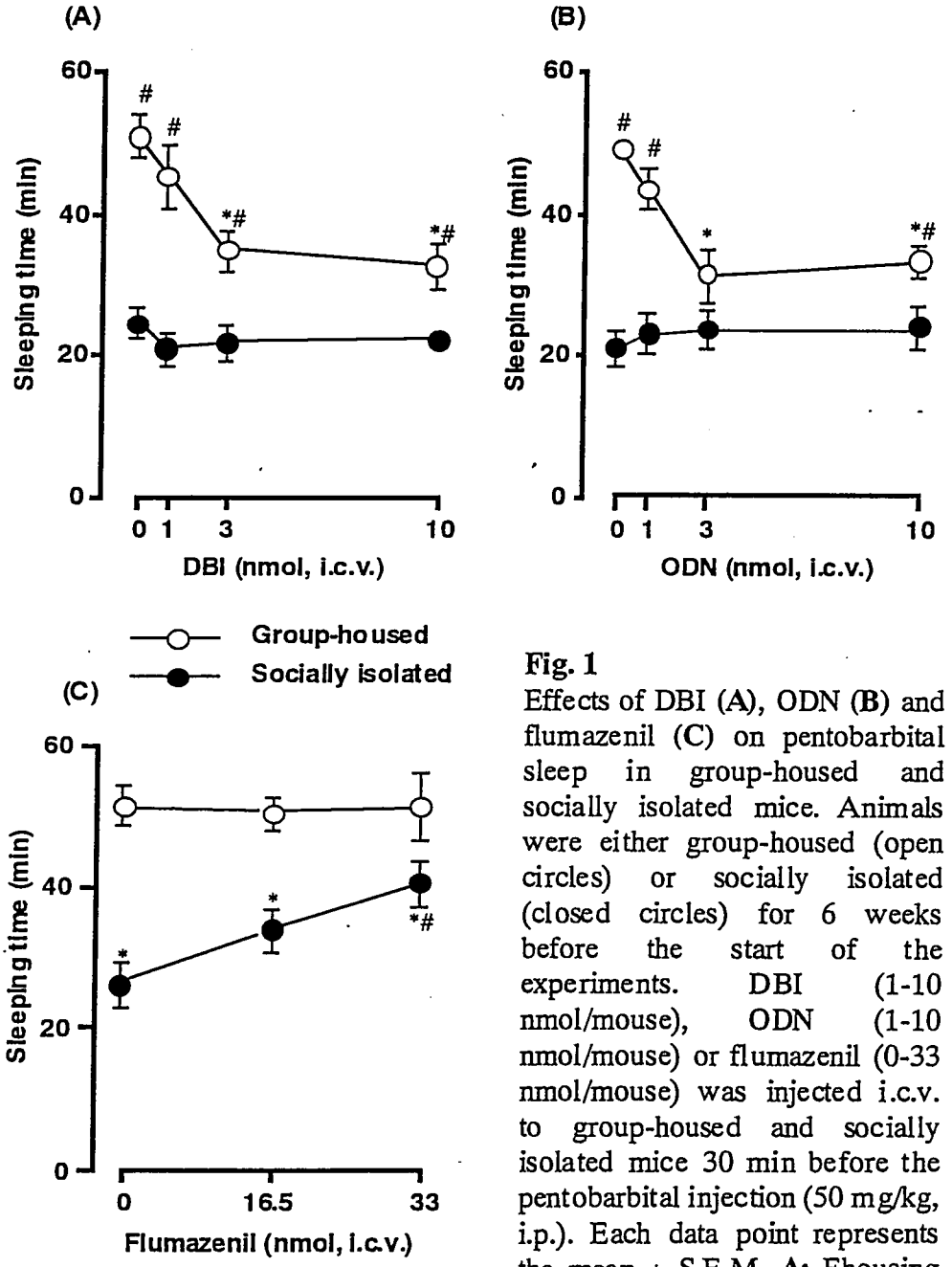

Fig. 1
Effects of DBI (A), ODN (B) and flumazenil (C) on pentobarbital sleep in group-housed and socially isolated mice. Animals were either group-housed (open circles) or socially isolated (closed circles) for 6 weeks before the start of the experiments. DBI (1-10 nmol/mouse), ODN (1-10 nmol/mouse) or flumazenil (0-33 nmol/mouse) was injected i.c.v. to group-housed and socially isolated mice 30 min before the pentobarbital injection (50 mg/kg, i.p.). Each data point represents the mean ± S.E.M. **A:** Fhousing conditions x DBI (3,69)=4.11, $P<0.01$. **B:** Fhousing conditions x ODN (3,71)=5.20, $p<0.01$. **C:** Fhousing condition x flumazenil (2,58)=38.24, $P<0.001$. *$p<0.05$ vs. respective group-housed animals. #$p<0.05$ vs. vehicle-treated control. Each datum represents the mean ± S.E.M. (n=9-10).

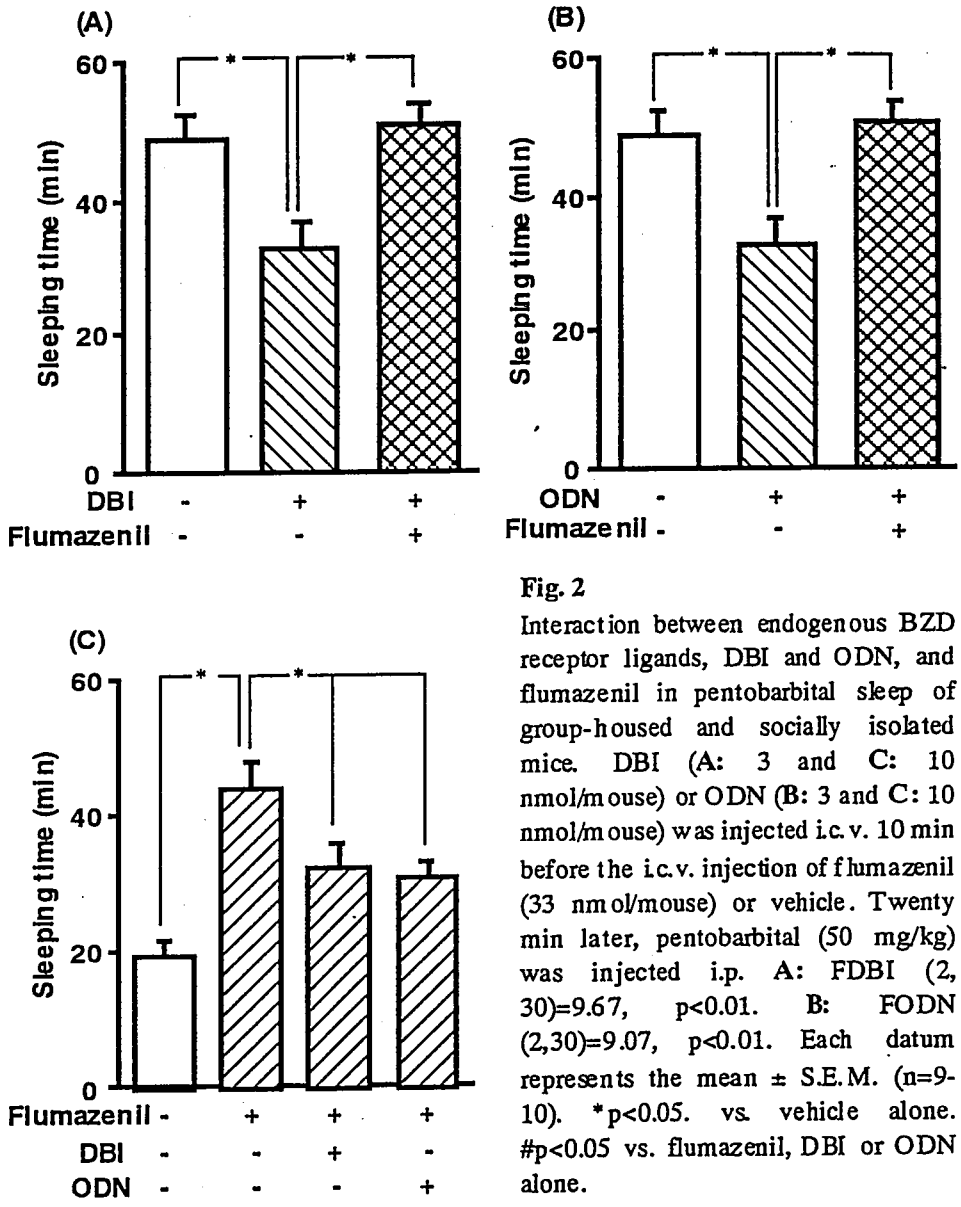

Fig. 2
Interaction between endogenous BZD receptor ligands, DBI and ODN, and flumazenil in pentobarbital sleep of group-housed and socially isolated mice. DBI (A: 3 and C: 10 nmol/mouse) or ODN (B: 3 and C: 10 nmol/mouse) was injected i.c.v. 10 min before the i.c.v. injection of flumazenil (33 nmol/mouse) or vehicle. Twenty min later, pentobarbital (50 mg/kg) was injected i.p. A: $F_{DBI}(2, 30)=9.67$, $p<0.01$. B: $F_{ODN}(2,30)=9.07$, $p<0.01$. Each datum represents the mean ± S.E.M. (n=9-10). *$p<0.05$. vs. vehicle alone. #$p<0.05$ vs. flumazenil, DBI or ODN alone.

Section II. Involvement of Peripheral Type of Benzodiazepine Receptor in Social Isolation Stress-induced Decrease in PB Sleep in Mice [2)]

In contrast to CBR, peripheral-type BZD receptors (PBR) located on the mitochondrial membrane of glial cells exerts an indirect action on $GABA_A$-R function through regulating neurosteroidogenesis. To clarify the role of PBR in the decrease in PB sleep caused by social isolation, FGIN-1-27 (FGIN) and PK 11195 (PK), PBR agonist

and antagonist, and pregnenolone (PREG), a neurosteroid precursor, and pregnenolone sulfate (PS), a neurosteroidal negative allosteric modulator of $GABA_A$-R were given i.c.v. 30 min before PB, respectively. FGIN, (25-100 nmol), PK (14-28 nmol), and PREG (15-30 nmol) dose-dependently normalized PB sleep in isolated mice without affecting that in group-housed animals. In contrast, PS (24 nmol) reduced the PB sleep in group-housed but not isolated mice. PS, at the same dose, significantly attenuated the effects of FGIN (100 nmol), PK (28 nmol) and PREG (30 nmol) in isolated mice, while FGIN (100 nmol), PK (28 nmol) and PREG (30 nmol) significantly blocked the effect of PS (24 nmol) in group-housed mice. These results suggest that decrease in the genesis of neurosteroid(s) with a $GABA_A$-R agonistic profile regulated by PBR is also partly implicated in the down regulation of $GABA_A$-R following social isolation and contributes to the decrease of PB-induced sleep in isolated mice. Besides, FGIN and PK, at the doses tested, tended to attenuate the DBI or ODN-induced decrease in PB sleep in group-housed mice, suggesting that these peptides preferentially interact with CBR in the brain as shown in Fig.3-7.

Section III. Benzodiazepine Binding Inhibitor (DBI) Gene Expression in The Brains of Socially Isolated and Group-housed Mice [3)]

To find biochemical evidence that supports behavioral findings descried above, DBI gene expression in the brain of group-housed and isolated mice was examined. Consistent with the previous reports, the *in situ* hybridization result showed strong signals of DBI mRNA around the regions of the third ventricle, especially the lining cells, the arcuate nucleus of the hypothalamus and the cerebellum in both socially isolated and group-housed animals. Unexpectedly, however, semi-quantitative analysis with RT-PCR technique revealed that isolated mice had significantly less expression of DBI mRNA in the hypothalamus than group-housed animals and there was no difference in the expression in the other brain areas between two groups. In addition, the significant decrease in the gene expression was found in the mice individually isolated for 2 weeks and this decrease paralleled with the decrease in PB sleep in the mice isolated for the same period. Although this finding hardly agrees with our hypothesis that DBI gene might be overexpressed in isolated mice, it provides evidence that isolation stress affects DBI gene expression. The most possible mechanism underlying this decrease of DBI mRNA is the

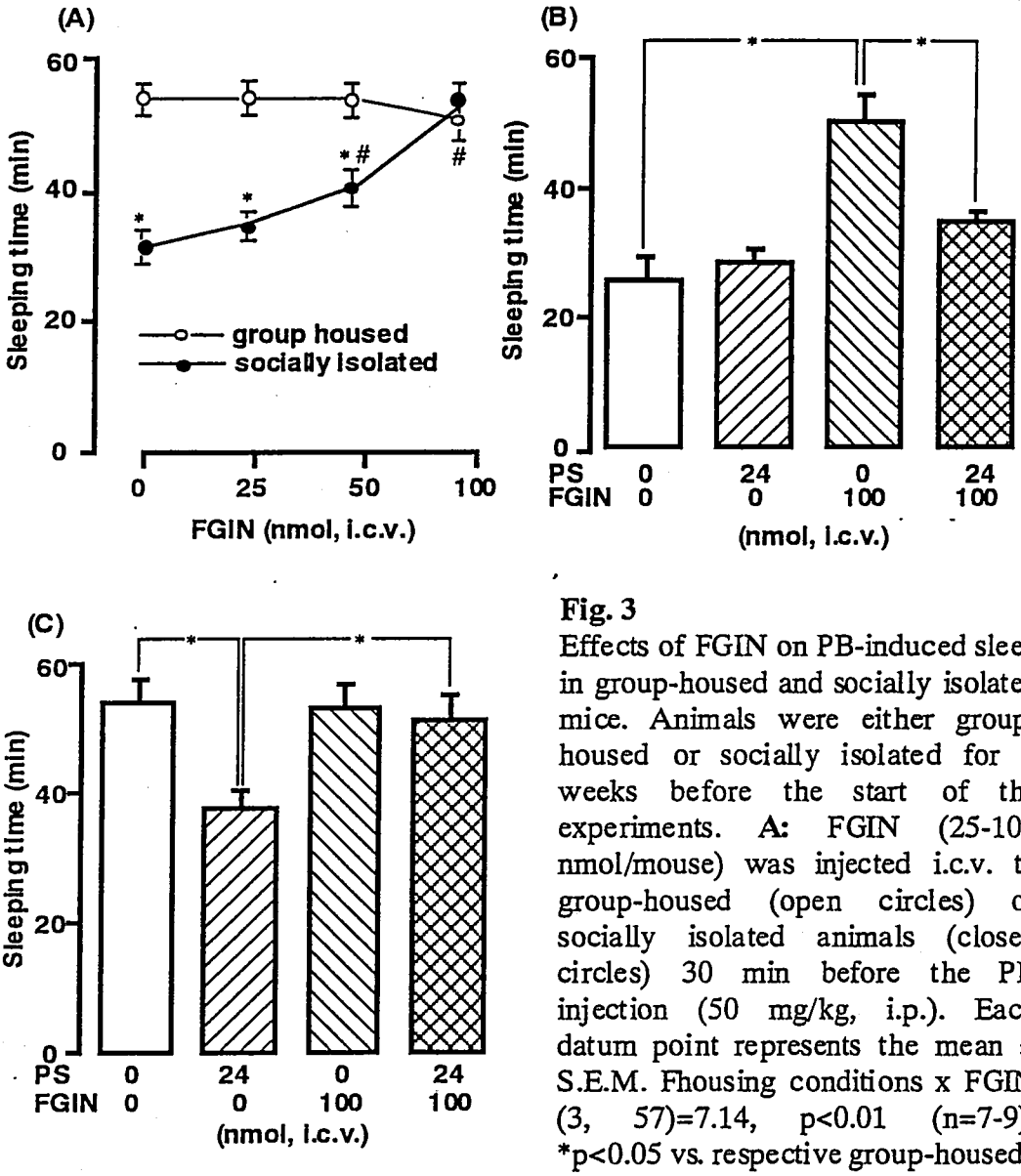

Fig. 3

Effects of FGIN on PB-induced sleep in group-housed and socially isolated mice. Animals were either group-housed or socially isolated for 6 weeks before the start of the experiments. **A:** FGIN (25-100 nmol/mouse) was injected i.c.v. to group-housed (open circles) or socially isolated animals (closed circles) 30 min before the PB injection (50 mg/kg, i.p.). Each datum point represents the mean ± S.E.M. Fhousing conditions x FGIN (3, 57)=7.14, $p<0.01$ (n=7-9). *$p<0.05$ vs. respective group-housed animals. #$p<0.05$ vs. vehicle-treated control. **B** and **C:** PS (24 nmol/mouse) and FGIN (100 nmol) were either given alone or coadministered i.c.v. to socially isolated mice (**B**) or group-housed mice (**C**) 30 min before PB (50 mg/kg. i.p.). Each datum represents the mean ± S.E.M. of 9-10 mice (**B**) or 8-9 mice (**C**), *$p<0.05$.

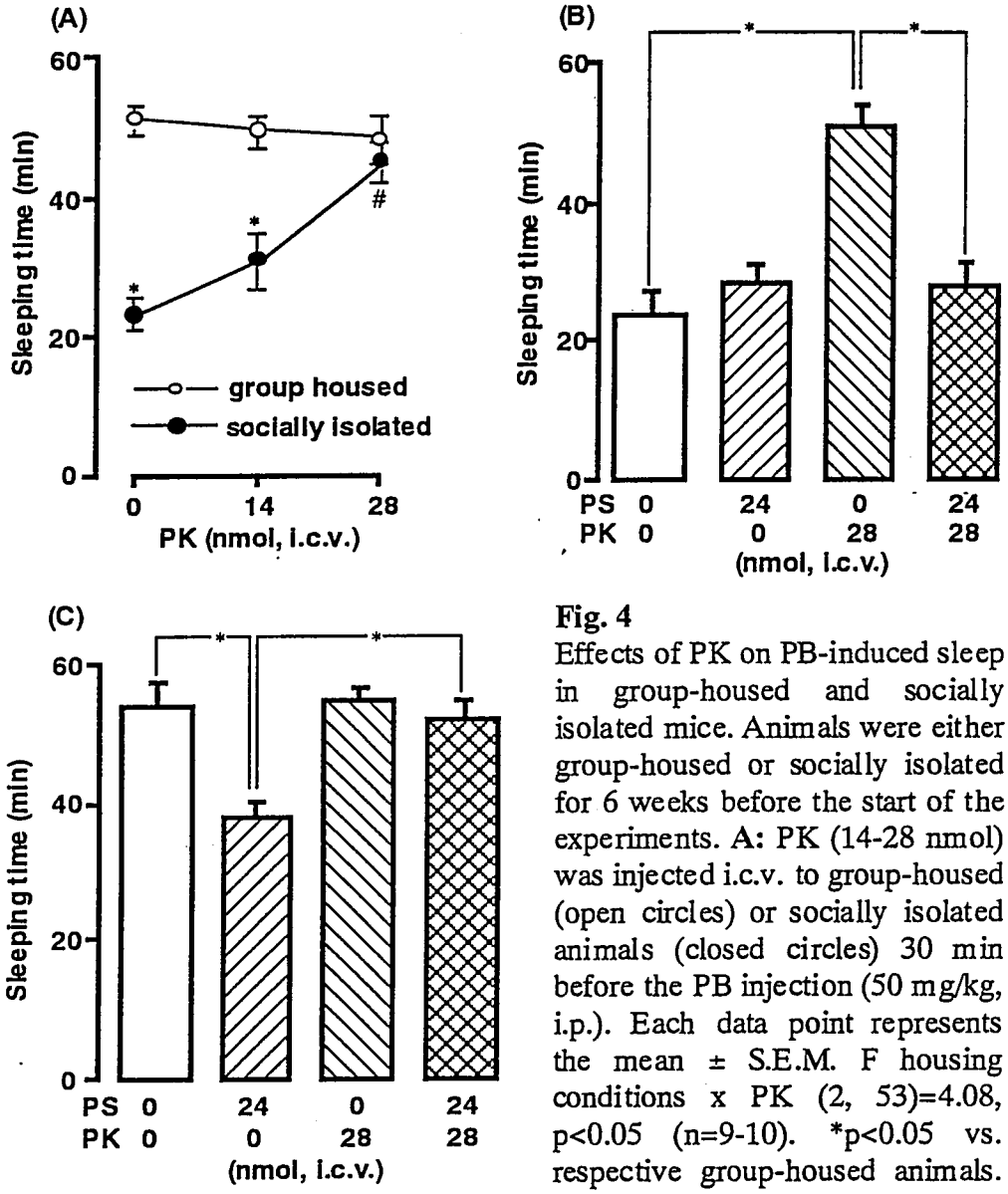

Fig. 4

Effects of PK on PB-induced sleep in group-housed and socially isolated mice. Animals were either group-housed or socially isolated for 6 weeks before the start of the experiments. **A:** PK (14-28 nmol) was injected i.c.v. to group-housed (open circles) or socially isolated animals (closed circles) 30 min before the PB injection (50 mg/kg, i.p.). Each data point represents the mean ± S.E.M. F housing conditions x PK (2, 53)=4.08, $p<0.05$ (n=9-10). *$p<0.05$ vs. respective group-housed animals. #$p<0.05$ vs. vehicle-treated control. **B** and **C**: PS and PK (24 and 28 nmol) were coadministered i.c.v. to socially isolated mice (**B**) or group-housed mice (**C**) 30 min before PB (50 mg/kg). Each datum point represents the mean ± S.E.M of 9 mice (**B**) or 8-9 (C), *$p<0.05$.

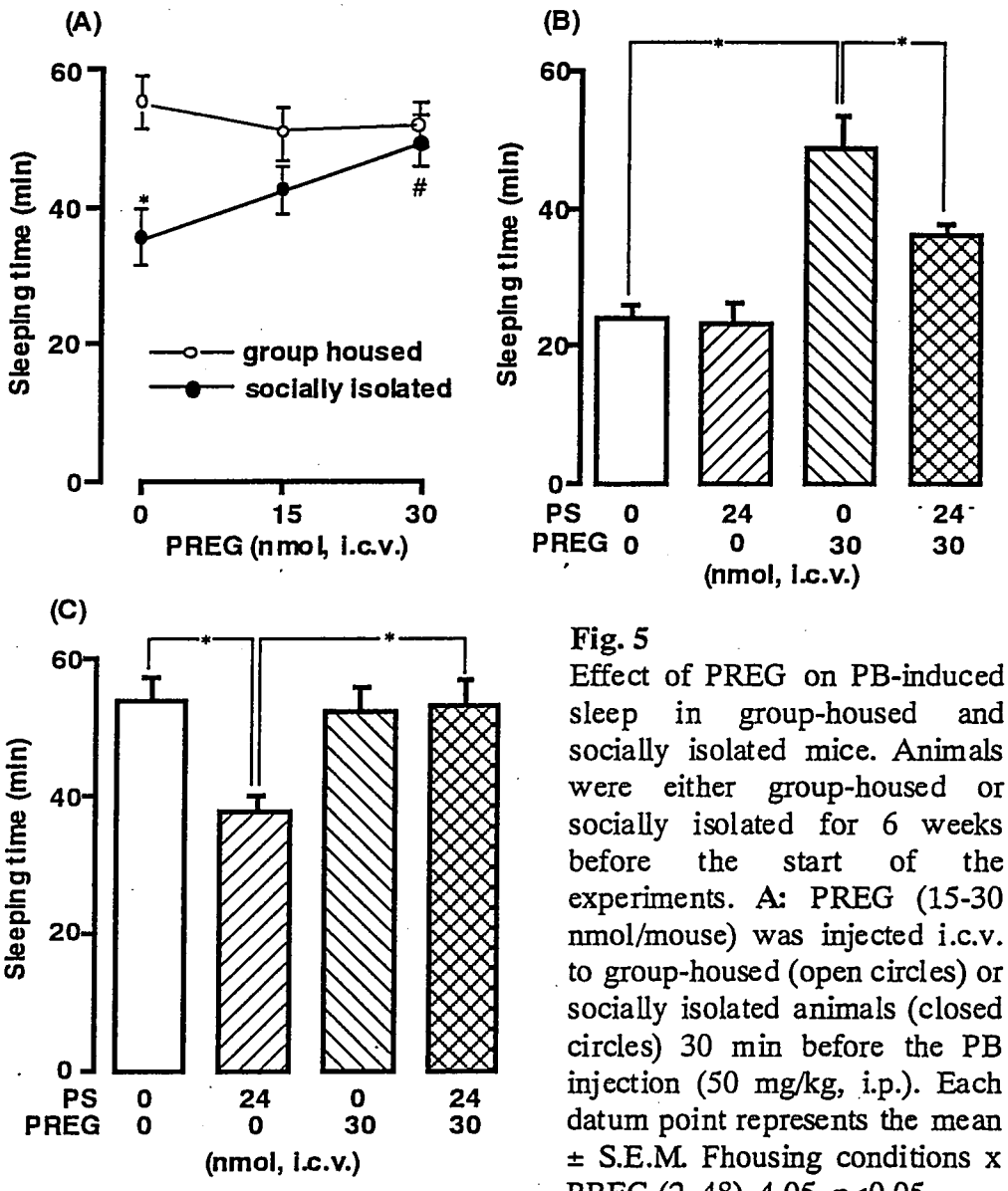

Fig. 5

Effect of PREG on PB-induced sleep in group-housed and socially isolated mice. Animals were either group-housed or socially isolated for 6 weeks before the start of the experiments. **A:** PREG (15-30 nmol/mouse) was injected i.c.v. to group-housed (open circles) or socially isolated animals (closed circles) 30 min before the PB injection (50 mg/kg, i.p.). Each datum point represents the mean ± S.E.M. Fhousing conditions x PREG (2, 48)=4.05, $p<0.05$ (n=9). *$p<0.05$ vs. respective group-housed animals. #$p<0.05$ vs. vehicle-treated isolated animals. **B** and **C:** PREG and PS (30 and 24 nmol) were coadministered to socially isolated mice (**B**) or group-housed mice (**C**) 30 min before PB (50 mg/kg). Each datum point represents the mean ± S.E.M of 9 mice (**B**) or 8-9 mice (**C**), *$p<0.05$.

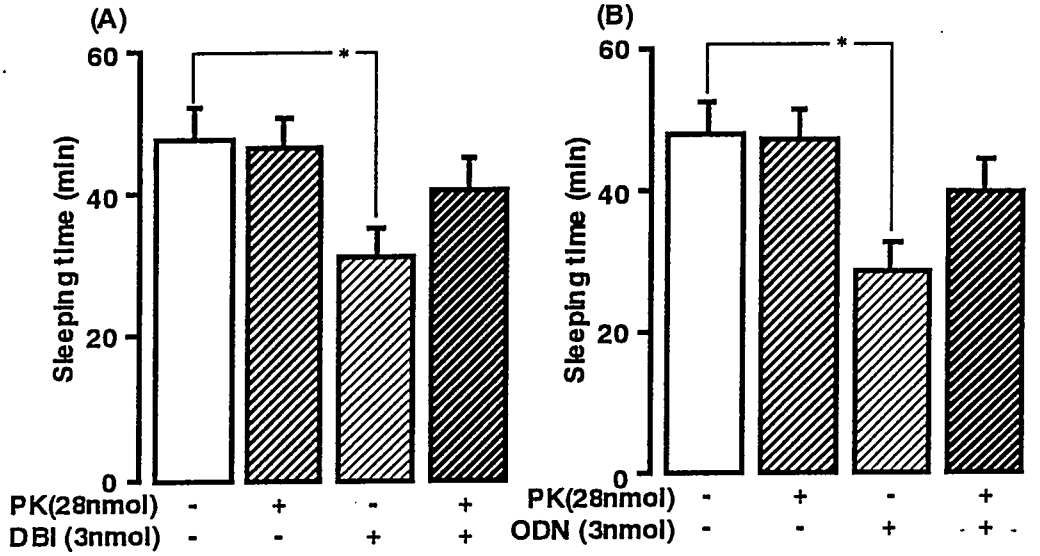

Fig.6 (A) and (B) Effects PK11195 (PK) on pentobarbital sleep in group-housed mice pretreated with DBI or ODN. Animals were either group-housed for 6 weeks before the start of the experiments. DBI or ODN (3nmol) was injected i.c.v. respectively 10 min before PK (28 nmol/mouse) given and then 20 min later the pentobarbital injection (50 mg/kg, i.p.) was administered. Each datum point represents the mean ± S.E.M (n=9-10). *p<0.05 vs. vehicle treated group.

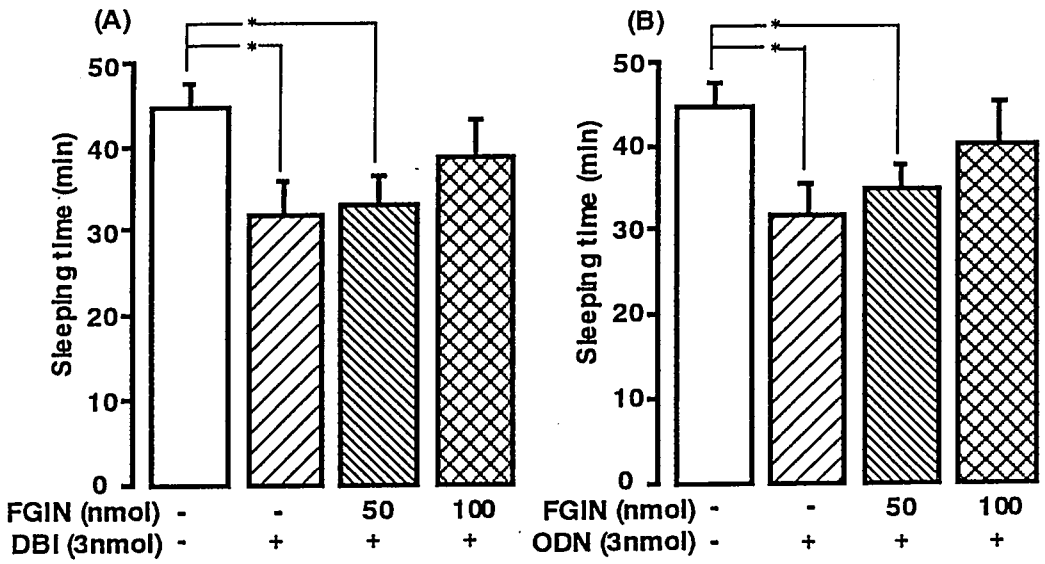

Fig.7 (A) and (B) Effects of FGIN-1-27 (FGIN) on pentobarbital sleep in group-housed mice pretreated with DBI or ODN. Animals were group-housed for 6 weeks before the start of the experiments. DBI or ODN (3nmol) was injected i.c.v. respectively 10 min before FGIN (50 and 100 nmol/mouse) given and then 20 min later the pentobarbital injection (50 mg/kg, i.p.) was administered. Each datum point represents the mean ± S.E.M (n=9-10). *p<0.05 vs. vehicle treated group.

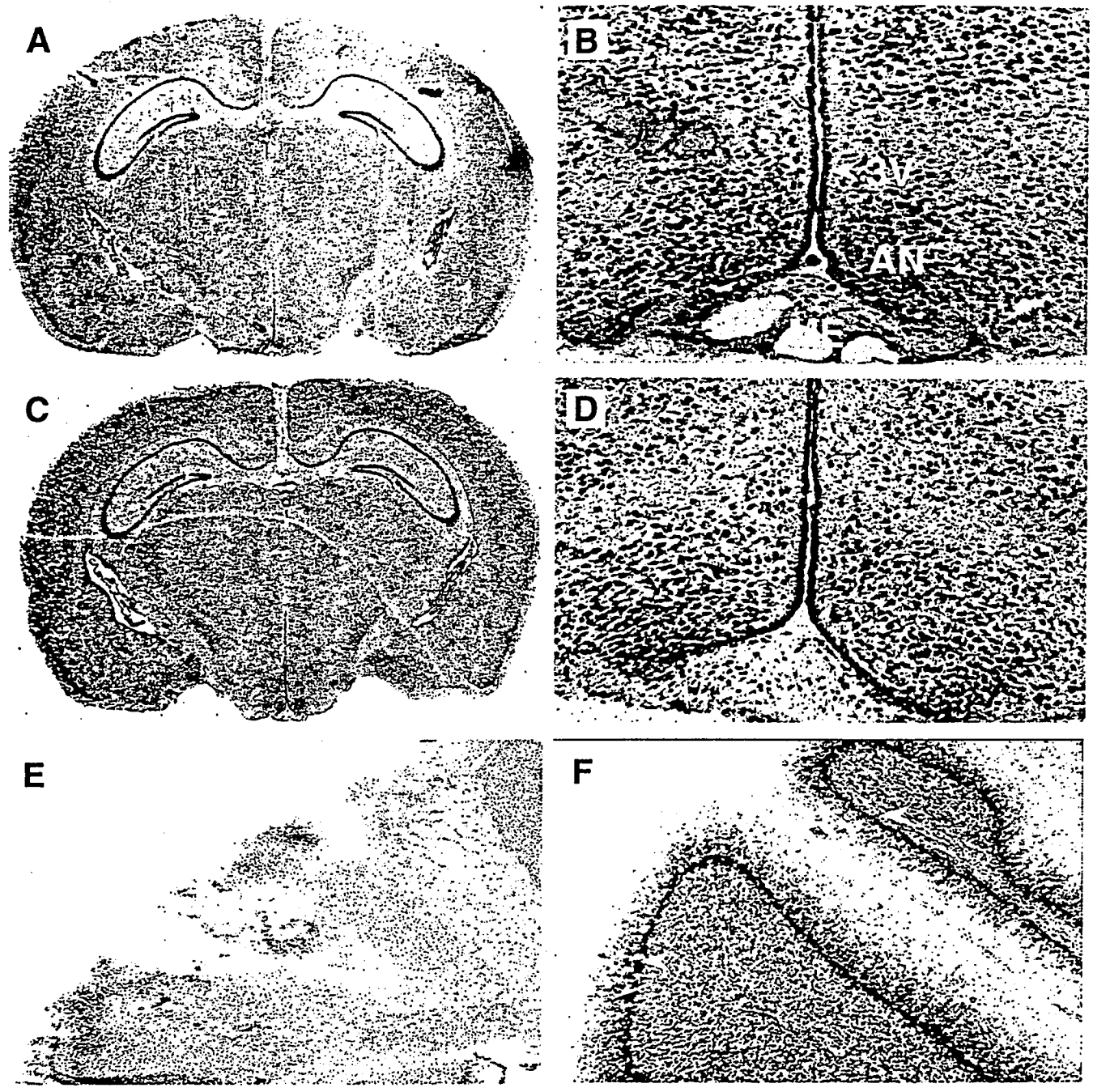

Fig.8

DBI mRNA distribution in the brain tissue of mice group-housed for 10 weeks. DBI mRNA was found around regions of the hypothalamus and cerebellum. A: Brain slice hybridized by DBI sense probe; B: Hypothalamus region hybridized by the sense probe, in which 3V, AN and ME represent the third ventricle (ependymal cells), arcuate nucleus and median eminence (10 x), respectively. Whole brain slice (C) and hypothalamus section (D) were prepared from group-housed mice and hybridized by DBI antisense probe. E and F: Cerebellum regions hybridized by sense probe (E), or antisense probe (F), respectively. DBI mRNA was found abundantly in the Purkinje layers (arrows, 10 x).

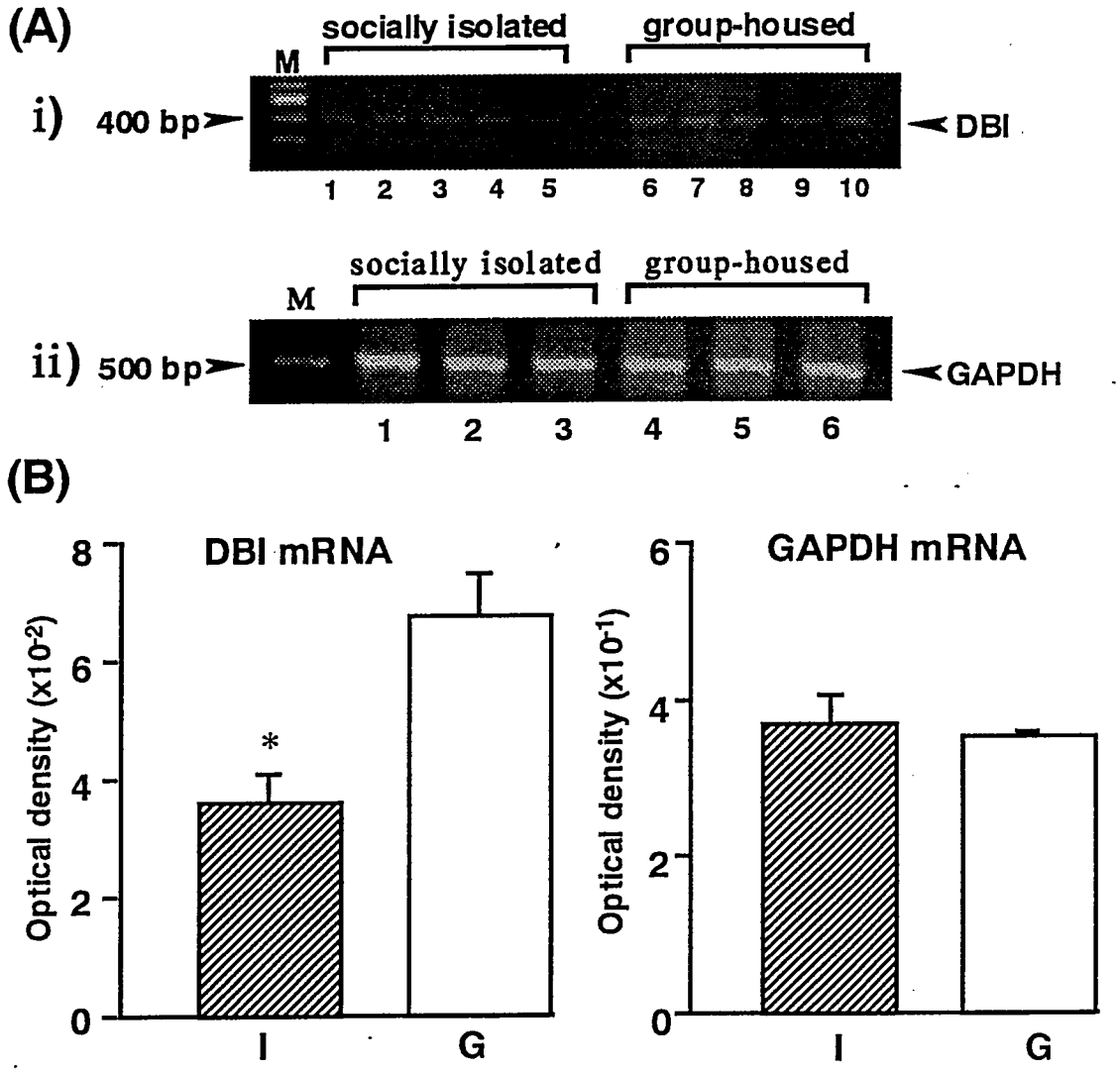

Fig. 9 Social isolation stress-induced changes in the DBI gene level in the brain. Mice were either group-housed or individually isolated for 6 weeks before the start of the experiments. DBI mRNA was extracted from the brains of each animal groups. **A:** digitized image of DBI mRNA (i, lanes 1-5: socially isolated mice; lanes 6-10: group-housed mice) and GAPDH mRNA (ii, lanes 1-3: socially isolated mice; lanes 4-6: group-housed mice) extracted from the hypothalamus region of socially isolated and group-housed mice. **B:** quantification of DBI and GAPDH mRNA signals in the hypothalamus region of group-housed and socially isolated mice. Each data column represents the mean ± S.E.M. (n=3-5). *P<0.001 (Student's *t* -test).

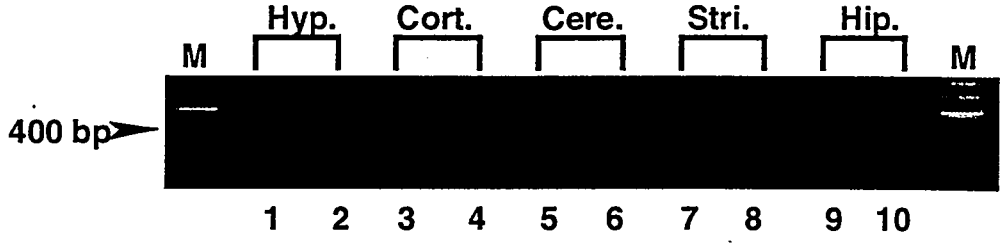

Fig. 10 Social isolation stress-induced changes in the DBI gene level in the brain. Mice were either group-housed or individually isolated for 6 weeks before the start of the experiments. DBImRNA was extracted from the brains of eachanimal groups. PCR image of DBI mRNA expressed in thehypothalamus (Hyp.), cortex (Cort.), cerebellum (Cere.), striatum (Stri.), and hippocampus (Hip.) of group-housed and socially isolated mice. DBI mRNA extracted from each brain region of socially isolated (lanes 1, 3, 5, 7, and 9) and group-housed mice (lanes 2, 4, 6, 8,and 10) was amplified as described in the text.

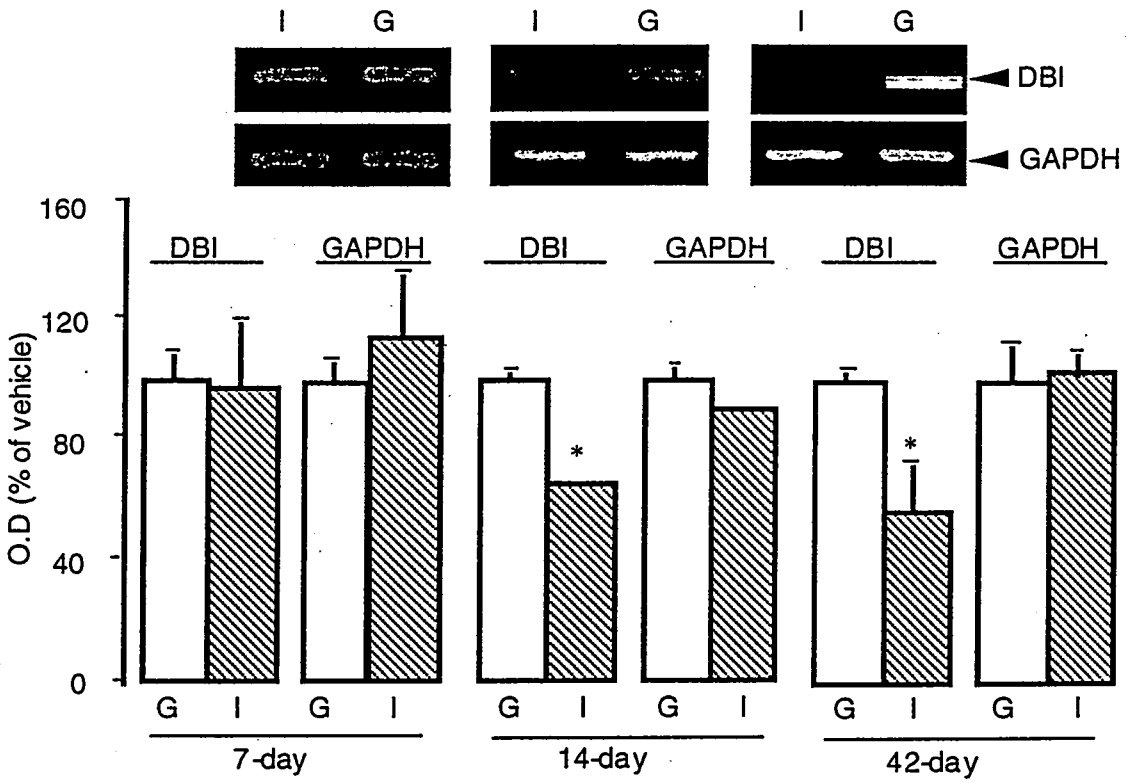

Fig. 11 Isolation stress time-dependently induces DBI gene level decrease in mice hypothalamic region

negative feedback regulation by DBI peptide, other endogenous substance(s) with more potent activity than DBI, or stress-related hormones (see Fig.8-11).

Section IV. Corticosterone Modulation in Hypothalamic DBI Gene Expression in Mice [4)]

Since the hypothalamo-pituitary-adrenocortical axis is promptly activated in response to variety of stressful stimuli and is duly responsible for the secretion of substantial quantities of glucocorticoids, it raises the possibility that glucocorticoids may be one of the factors that can affect DBI gene expression in the brain. To elucidate this hypothesis, male ddY mice (4 weeks old) received stress-dose of corticosterone (CORT, 1 or 5 mg, s.c.) daily for 1, 3 and 7 days and DBI mRNA level in the hypothalamus and the duration of PB sleep in these animals were measured. One day after the single injection of CORT (1 and 5 mg), 5 but not 1 mg CORT significantly decreased DBI gene level in the hypothalamus. When the treatment was repeated for 3 or 7 days, the remarkable reduction in DBI gene level appeared in the animals injected with 1 mg CORT. In parallel with the change in the gene level, PB-induced sleep also decreased significantly in CORT-treated mice. These results suggest that the decrease in DBI mRNA level in isolated mice may be partly due to a negative regulation by CORT in the brain (as shown in Fig.12-14).

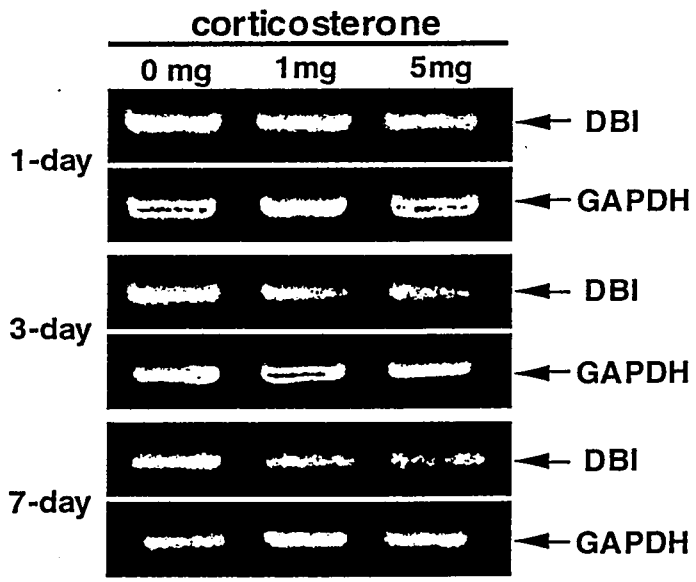

Fig. 12 Corticosterone-induced alteration in hypothalamic DBI gene level in mice. Mice were given s.c. corticosterone for 1, 3 and 7 days before the start of the experiments. RT-PCR images show the changes of DBI gene following certain interval administration of the drug. To avoid any deviation caused by sampling, GAPDH mRNA was used as a internal control.

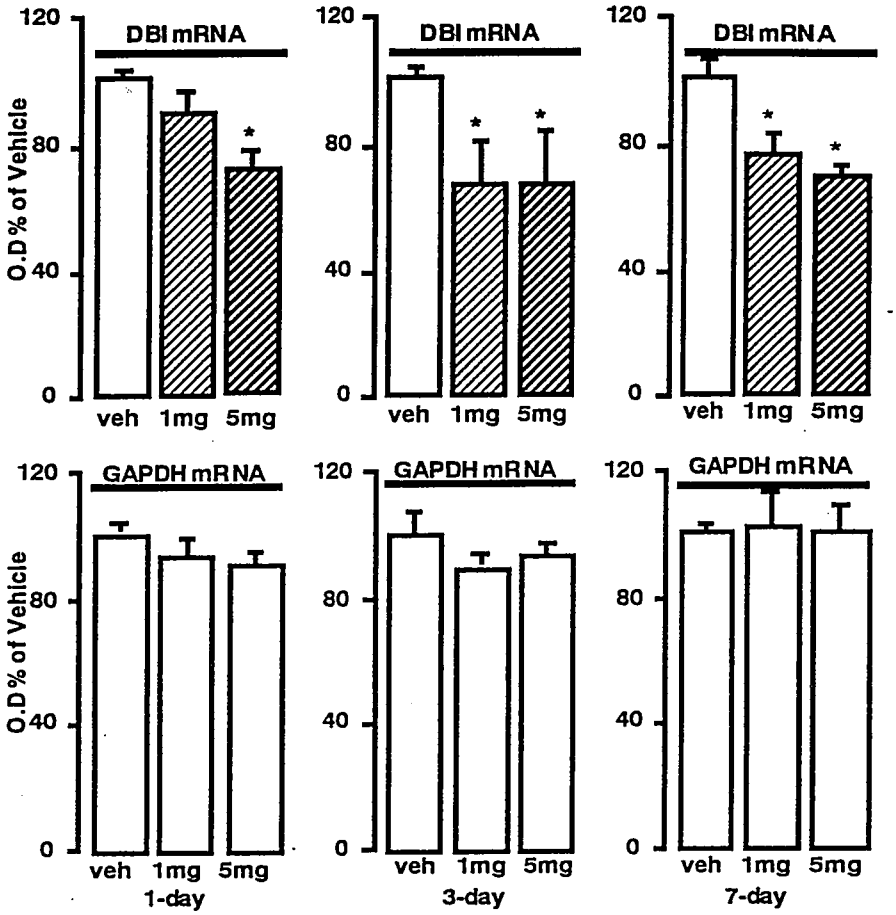

Fig. 13 Semiquantity of RT-PCR images of DBI and GAPDH mRNA signals in hypothalamic region. Each data column represents the mean ± S.E.M.(n=3). *p<0.05.

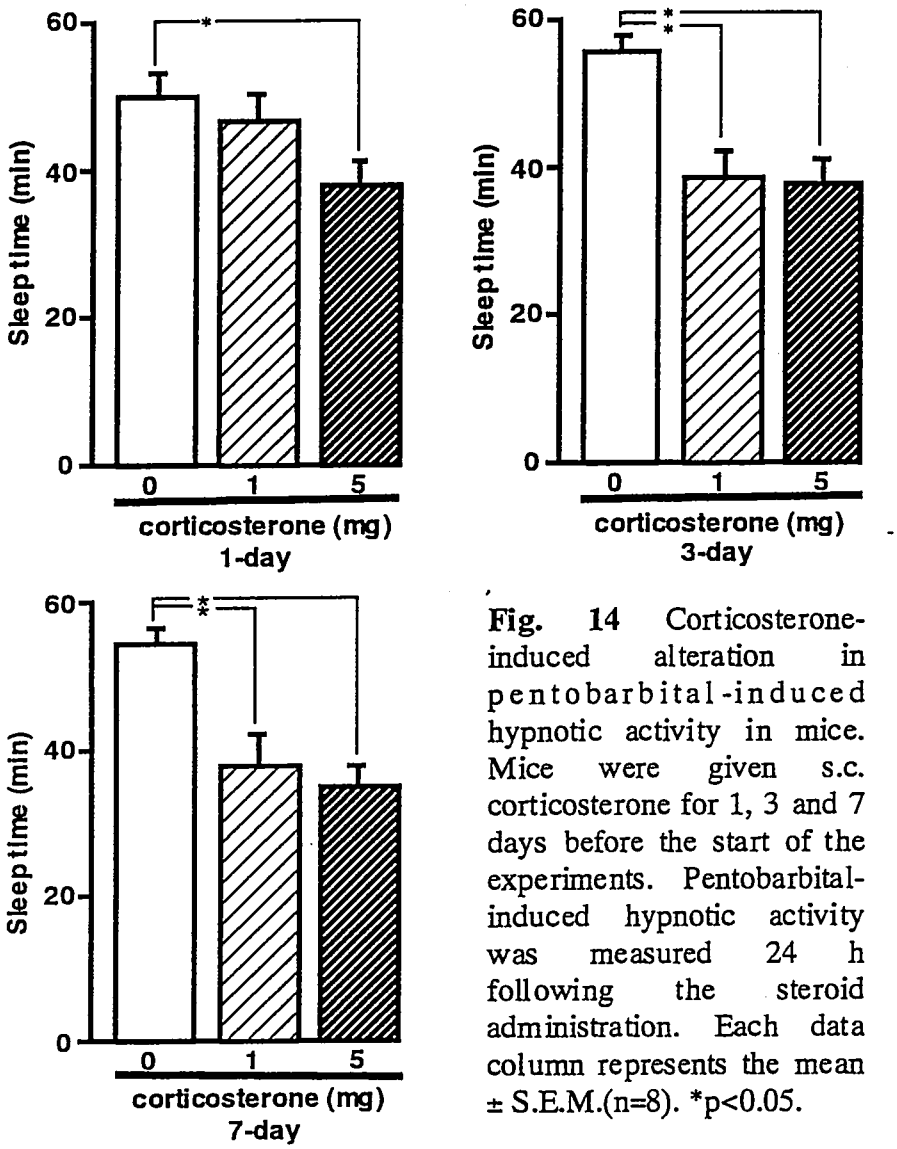

Fig. 14 Corticosterone-induced alteration in pentobarbital-induced hypnotic activity in mice. Mice were given s.c. corticosterone for 1, 3 and 7 days before the start of the experiments. Pentobarbital-induced hypnotic activity was measured 24 h following the steroid administration. Each data column represents the mean ± S.E.M.(n=8). *p<0.05.

General Conclusion

The present findings have clarified that, 1) DBI, an endogenous BZD ligand, is responsible for the decrease in PB sleep in social isolation mice through directly and indirectly down-regulating $GABA_A$-R function by binding to CBR and PBR, 2) isolation stress induces a decrease in hypothalamic DBI gene level, possibly through a negative-feedback mechanism by which CORT may act as an important modulator.

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Diazepam binding inhibitor (DBI) gene expression in the brains of socially isolated and group-housed mice

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Abstract

Diazepam binding inhibitor (DBI), a putative endogenous polypeptide ligand for benzodiazepine (BZD) receptors, has been shown to act as an inverse BZD receptor agonist in the brain. We previously suggested that the social isolation stress-induced decrease in pentobarbital sleeping time in mice was partly due to an increase in the activity of endogenous substances with an inverse BZD receptor agonist-like property such as DBI. In this study, we examined whether the DBI gene expression is affected by socially isolated stress. Consistent with the previous findings, the in situ hybridization result showed very strong signals of DBI mRNA around the regions of the third ventricle, especially the lining cells, the arcuate nucleus of the hypothalamus and the cerebellum, in both socially isolated and group-housed animals. Unexpectedly, however, semi-quantitative experiments with reverse transcription–polymerase chain reaction technique revealed that socially isolated mice had significantly less expression of DBI mRNA in the hypothalamus than group-housed animals, and no difference in the expression in the other brain areas was observed between two animal groups. We discuss the relationship between the decrease of DBI mRNA expression in the hypothalamus and the decrease of $GABA_A$ receptor function following long-term social isolation in mice.

Keywords: DBI mRNA; Diazepam binding inhibitor (DBI); In situ hybridization; RT-PCR; Social isolation stress

1. Introduction

Our previous studies have showed that the pentobarbital-induced sleeping time in socially isolated mice is significantly shorter than that in group-housed animals and the intracerebroventricular (i.c.v.) injection of the benzodiazepine (BZD) receptor antagonist flumazenil can normalize the pentobarbital-induced sleeping time in isolated mice without affecting the sleep in group-housed animals (Ojima et al., 1995; Matsumoto et al., 1996; Ojima et al., 1997). In addition, the effect of an inverse BZD receptor agonist FG7142 on pentobarbital sleep in group-housed and socially isolated mice was opposite to that of flumazenil; FG7142 caused a decrease in the sleeping time in group-housed mice without changing the sleep in socially isolated mice. Based on these findings we hypothesized that endogenous substance(s) with an inverse BZD receptor agonist property may be involved in this alteration (Ojima et al., 1997).

Diazepam binding inhibitor (DBI) and its spliced fragment, octadecaneuropeptide (ODN) and triakontatetraneuropeptide (TTN) are putative endogenous BZD receptor ligands (Guidotti et al., 1983; Guidotti, 1991). Evidence indicates that these peptides negatively modulate the $GABA_A$ receptor function by binding to BZD recognition sites (Guidotti et al., 1983) like an inverse BZD receptor agonist, and that their effects on $GABA_A$ receptors are reversed by flumazenil (Costa, 1991; Costa and Guidotti, 1991). Recently we found that DBI shortened the pentobarbital sleeping time in

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group-housed but not in socially isolated mice when given i.c.v. and that flumazenil reversal of pentobarbital sleep in socially isolated mice was attenuated by the i.c.v. injection of DBI (Dong et al., 1998). These findings raise the possibility that DBI plays an important role in the reduction of barbiturate-induced sleep by long-term social isolation. Thus, in the present study, we examined the changes in DBI mRNA expression in the brains of mice following long-term social isolation by an in situ hybridization technique and a reverse transcription–polymerase chain reaction (RT-PCR) method.

2. Materials and methods

2.1. Animals

Male ddY mice, weighing 18–20 g (Japan SLC, Shizuoka, Japan) were obtained at the age of 28 days. Animals were either housed in groups of five per cage (24 × 17 × 12 cm) or individually isolated in the same size cage. In the in situ hybridization experiments, the animals housed in groups or individually isolated for 2, 4, 6, 8 and 10 weeks were used. For the RT-PCR of DBI mRNA, the animals housed in groups or individually isolated for 6 weeks were used. The housing was thermostatically maintained at 24 ± 1°C with constant humidity (65%) and a 12:12 h light/dark cycle (lights on: 07:00–19:00). The mice were given free access to food and water. The present studies were conducted in accordance with the standards established by the Guide for the Care and Use of Laboratory Animals of Toyama Medical and Pharmaceutical University.

2.2. In situ hybridization

The DBI cDNA was isolated from the mouse brain mRNA by using the RT/PCR method. The sequences of the sense and the antisense primers are ATTCGGCATCCGTATCAC and AGCAGAGGTTAACGCTGGCCCTAAT which correspond to the sequences from 13 to 29 and from 393 to 417, respectively (Owens et al., 1989). The resultant purified 405-bp fragment was inserted to the pBluescript II KS (−) vector which was treated with *Eco*RV at 37°C for 2 h followed by *Taq* polymerase with dTTP at 70°C for 2 h. The vector pDBI was cut by *Bam*HI at nucleotide 692 of DBI cDNA for a T3 RNA polymerase reaction to make antisense RNA, and by *Sal*I at nucleotide 657 of the vector for a T7 RNA polymerase reaction to make sense RNA. The reactions to make these probes were performed essentially as described previously (Tohda and Watanabe, 1996). After incubation at 37°C for 60 min, the reaction mixtures were further treated with 0.1 mg/ml DNaseI for 15 min, and the reaction mixture was then precipitated with ethanol in the presence of 0.4 M LiCl and 25 mM EDTA. Both the digoxigenin-labeled antisense (T3) and sense (T7) cRNA probes had an apparent length of about 500 bp. The resultant cRNA was placed into hydration buffer (80 mM $NaHCO_3$, 60 mM Na_2CO_3, 6 mM dithiothreitol) and incubated at 60°C to generate fragments of about 200–300 bp. The reaction was terminated by adding 0.3 M sodium acetate and 250 μg tRNA, and then precipitated with ethanol. The pellet was re-dissolved in sterile water and stored at −80°C. In in situ hybridization experiments, the mice were killed by decapitation and the brain was removed, immediately frozen with powdered dry ice, and stored for 1 day at −80°C. Frozen brain sections (16 μm thick) with the coordinates of A 2.3 mm from the frontal zero plane according to the atlas of Montemurro and Dukelow (1972) were cut using a cryostat, mounted onto gelatin-coated slides, and air dried. Before hybridization, sections were fixed with 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) for 15 min, followed by proteinase K treatment (0.5 μg/ml) and acetylation. The sections were dehydrated through a graded ethanol series, placed in chloroform to remove fat, treated with 100% ethanol and dried. After prehybridization, the sections were hybridized overnight with a digoxigenin-labeled probe in a hybridization buffer consisting of 5 × standard saline citrate (SSC: 150 mM NaCl, 17 mM sodium citrate, pH 7.0), 50% formamide, 2.7 × Denhardt's solution, 10 mM EDTA, 20 mM dithiothreitol, 0.25 mg/ml tRNA and 10% dextran sulfate at 55°C. The hybridized sections were washed with 2 × SSC at 55°C, treated with 50 μg/ml RNaseA for 30 min, washed with 50% formamide/2 × SSC at 55°C, dehydrated in ethanol and dried. DBI mRNA hybridized with the digoxigenin-labeled probe was detected immunohistochemically using an alkaline phosphatase (AP)-conjugated antidigoxigenin antibody with 450 μg/ml nitroblue tetrazolium and 175 μg/ml 5-bromo-4-chloro-3-indolyl-phosphate as substrates.

2.3. RT-PCR

Total RNA in hypothalami, cortices, cerebella, striata and hippocampi of socially isolated and group-housed mice was extracted with guanidium isothiocyanate according to the method of Sambrook et al. (1989). Briefly, first-strand cDNA was synthesized using 5 μg of total RNA from group-housed or socially isolated animals as a template and antisense probe of DBI as a primer; superscript reverse transcriptase was used for the PCR fingerprinting. In addition to the template, each PCR reaction contained 0.25 mM deoxynucleoside triphosphate mix, 5 μM primers, *Taq* polymerase, PCR buffer and 2.2 mM $MgCl_2$. The PCR subseries primers used were the antisense and sense sequences of

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DBI described above. Thermal cycling was performed using a thermal cycler (PCR Thermal Cycler MP, Takara, Tokyo, Japan) and the PCR mixture was amplified as follows: one cycle of 94°C for 5 min and then 12–16 cycles of 94°C for 1 min, 62°C for 2 min, and 72°C for 2 min. These conditions were evaluated by electrophoresis, and the curve of optical density of the band versus the number of cycles was run. The PCR products were electrophoresed on a 3% agarose gel and run in a buffer containing 1 mM EDTA, 40 mM Tris and 20 mM acetic acid (pH 8.0). The gels were then visualized by ethidium bromide staining. The optical densities of the bands appeared at 400 bp were determined using a densitometer (Bio-Rad, Richmond, CA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was also detected as internal control. The sequences of primer pairs of GAPDH were 5'-CCAAGGTCATCCATGACAAC-3' (sense) and 5'-TTACTCCTTGGAGGCCCATGT-3' (antisense). The PCR program for GAPDH mRNA was performed with 20 thermal cycles similarly to that of DBI. The GAPDH mRNA band appeared at about 500 bp.

2.4. Statistics

RT-PCR data from both animal groups were analyzed by Student's t-test. Differences with $P < 0.05$ were considered significant.

3. Results

In the in situ hybridization study, the sites of expression of DBI mRNA were visualized by the concentration of purple/blue AP reaction product on brain slices. In the brain coronal sections hybridized with the antisense probe, the highest signal (i.e. the areas of dense staining) was observed in the third ventricle lining cells (ependymal cells) of the hypothalamic region and Purkinje layers of the cerebellum in socially isolated and group-housed mice. In addition, the arcuate nucleus and median eminence showed considerably high levels of DBI mRNA compared with the respective sections hybridized with the control sense probe of DBI mRNA, while the cerebral cortex, the mesencephalic and telencephalic regions, the hippocampus and some thalamic nuclei exhibited a weak staining (Fig. 1). In some brain slices, a slight difference in DBI mRNA signal between the two animal groups was observed, but it was hard to evaluate the difference quantitatively (data not shown).

To compare in more detail the DBI mRNA expression in the brain between group-housed and socially isolated mice, a semiquantitative RT-PCR method was used. As shown in Fig. 2, the optical density of the DBI mRNA band was increased almost linearly by at least 16 cycles. Consistent with the data obtained by the present in situ hybridization experiments, the hypothalamus and cerebellum exhibited more marked signals of DBI mRNA compared to other brain regions in the group-housed and socially isolated mice. However, the comparison of the DBI mRNA expression in the hypothalamus revealed that the socially isolated mice exhibited significantly less DBI mRNA expression than the animals housed in groups for the same period, whereas both groups exhibited almost the same level of GAPDH mRNA (Fig. 3). In contrast, social isolation did not affect the expression of DBI mRNA in the cerebellum and the cerebral cortex (Fig. 3). The striatum and hippocampus exhibited bands of DBI mRNA that were so dim that the comparisons could not be made between both group animals.

4. Discussion

Our previous study suggested that social isolation stress-induced decrease in the hypnotic action of pentobarbital in mice is partly attributed to the suppression of $GABA_A$ receptor function, and that this suppression is mediated by an increase in the activity of endogenous BZD receptor ligand(s) with an inverse agonistic property at the $GABA_A$ receptor complex and/or the changes in neurosteroids capable of modulating the $GABA_A$ receptor function (Matsumoto et al., 1996; Ojima et al., 1997; Dong et al., 1998). Thus, we hypothesized that social isolation stress may change the DBI gene expression in the brain, since: (i) in vivo and in vitro studies have shown that DBI produces inverse BZD receptor agonistic actions via the BZD receptor on the $GABA_A$ receptor complex (Costa and Guidotti, 1991) and (ii) recent findings in our laboratory suggested that DBI played an important role in the decrease of pentobarbital sleep caused by social isolation stress (Dong et al., 1998).

To test this hypothesis, we first elucidated the localization of DBI gene in the brains of both group animals using in situ hybridization. The distribution pattern of DBI mRNA in the mouse brain agreed with the previous reports (Alho et al., 1985, 1988; Malagon et al., 1993), indicating that both sense and antisense primers synthesized in this study were the same as the ones reported (Owens et al., 1989). However, in situ hybridization results failed to clearly show the differences in DBI mRNA expression between the two groups of animals. In an attempt to quantitatively evaluate the DBI mRNA level, a RT-PCR technique was employed. Unexpectedly, the animals individually isolated for 6 weeks exhibited significantly less DBI mRNA expression in the hypothalamus than the animals housed in groups for the same period. These findings suggest a close relationship between the change in the DBI mRNA expression and the decrease in GABAergic function in socially isolated animals.

The present result showed that the effect of social isolation stress on DBI mRNA expression was not ubiquitous in the brain and the expression in the hypothalamus was found to be most susceptible to long-term social isolation stress compared to that in other brain areas. Although we cannot rule out the contribution of the DBI gene in the ependymal cells to the social isolation stress-induced change in the hypothalamic DBI mRNA content because the hypothalamic tissue used for DBI mRNA extraction was not separated from the ependymal cell layer, these findings are very interesting since the hypothalamus plays an important role in a variety of stress-related responses and DBI in this area has been suggested to have a physiological role in regulating the onset of behavioral patterns like anxiety, convulsions, aggression, etc. (Alho et al., 1985).

The unexpected results on DBI mRNA expression in the hypothalamus of socially isolated mice apparently conflict with our previous behavioral findings that endogenous BZD receptor ligands, such as DBI and/or ODN, capable of suppressing the $GABA_A$ receptor function may be overexpressed during a period of social isolation stress exposure to mice (Ojima et al., 1997; Dong et al., 1998). The exact reason for this discrepancy between present biochemical findings and previous behavioral results remains unclear, but the social isolation-induced decrease in the hypothalamic DBI

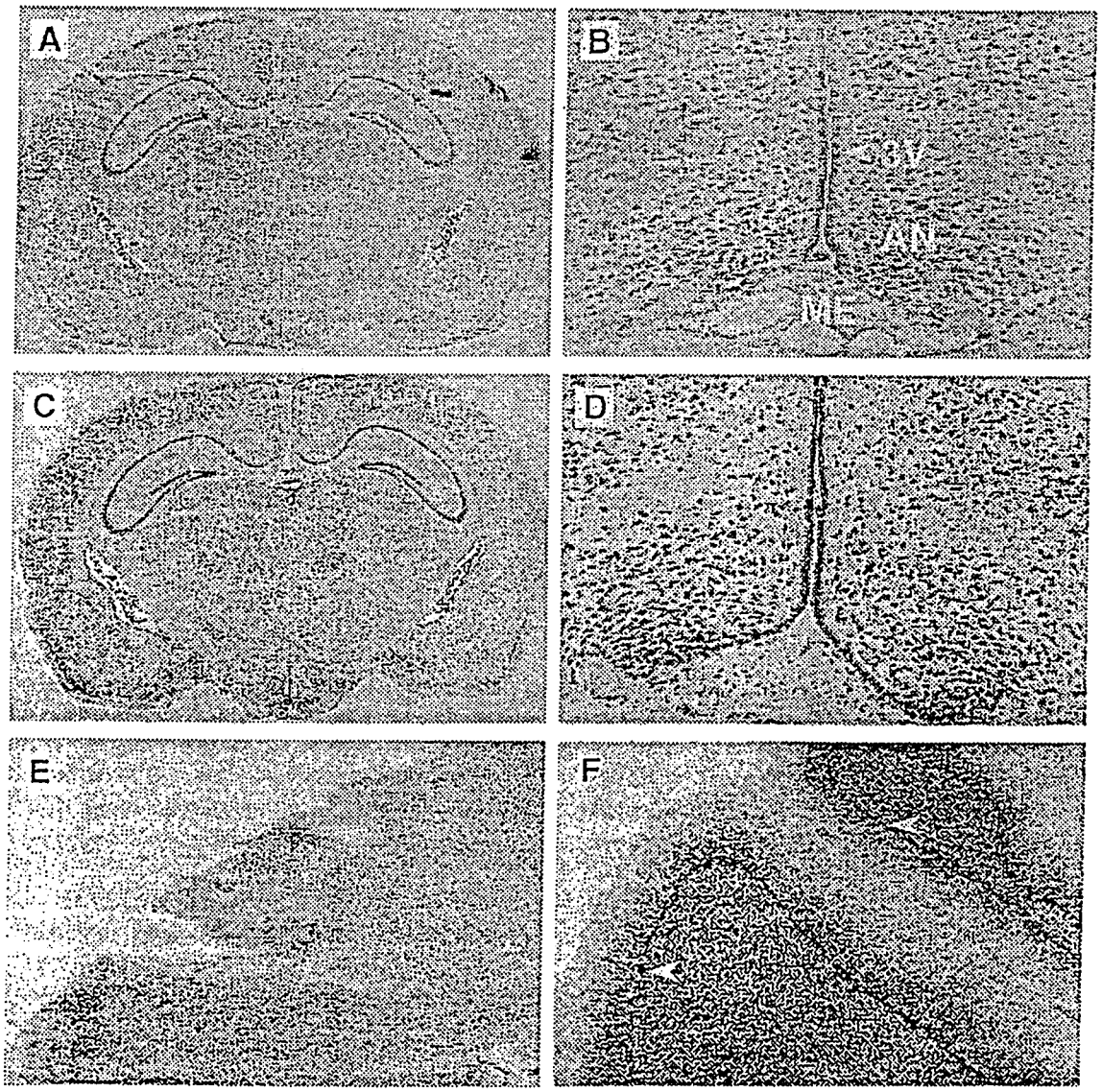

Fig. 1. DBI mRNA distribution in the brain tissue of mice group-housed for 10 weeks. DBI mRNA was found around regions of the hypothalamus and cerebellum. (A) Brain slice hybridized by DBI sense probe; (B) hypothalamus region hybridized by the sense probe, in which 3V, AN and ME represent the third ventricle (ependymal cells), arcuate nucleus and median eminence (10 ×), respectively. Whole brain slice (C) and hypothalamus section (D) were prepared from group-housed mice and hybridized by DBI antisense probe. (E, F) Cerebellum regions hybridized by sense probe (E), or antisense probe (F), respectively. DBI mRNA was found abundantly in the Purkinje layers (arrows, 10 ×).

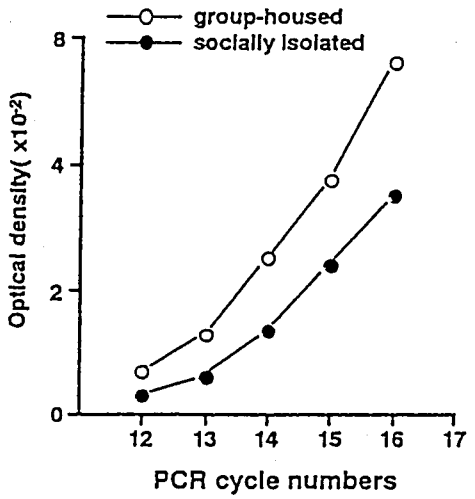

Fig. 2. Amplification kinetics of RT-PCR product (DBI gene) from the hypothalami of both group-housed and socially isolated mice. The thermal cycling was performed by the following program: 1 cycle of 94°C for 5 min, then 12–16 cycles at 94°C for 1 min, at 62°C for 2 min, and at 72°C for 2 min. Each data point represents the mean value of two independent experiments.

gene expression seems to be explained by several factors. First, there is a possibility that other endogenous substances with a more potent suppressive action on the $GABA_A$ receptor than DBI may be induced by social isolation stress and play an inhibitory role in DBI synthesis via a negative feedback mechanism. Several DBI fragments such as ODN and TTN can be generated by proteolytic cleavage of DBI and exert more potent activity at BZD receptors than DBI (Guidotti et al., 1983, 1986; Slobodyansky et al., 1989; Ferrarese et al., 1991). In addition, our previous study demonstrated that ODN produced the same action as DBI in terms of pentobarbital sleep in group-housed and socially isolated mice and attenuated the normalizing effect of flumazenil on pentobarbital sleep in socially isolated animals at the same dose range as DBI (Dong et al., 1998). Thus, it will be very interesting to test whether endogenous substance(s) with more potent inhibitor activity against $GABA_A$ receptors than DBI can be induced by the isolation stress.

Secondly, stress-related hormones such as corticotropin-releasing factor (CRF) and adrenocorticotropic hormone may be involved in the social isolation-induced decrease in DBI mRNA expression in the hypothalamus. We previously demonstrated that decrease of pentobarbital sleep caused by social isolation stress was partly due to hyperactivity of corticotropin-releasing factor systems in the brain (Ojima et al., 1995) and that this decrease was not attenuated by adrenalectomy (Matsumoto et al., 1996). Moreover, DBI has been suggested to have a facilitating role in CRF release through the $GABA_A$ receptor in the human brain (Roy et al., 1989). Considering the findings that elevated glucocorticoid hormones response to stress exert a negative feedback to prevent the nervous system from over-reacting to stressful stimuli (Munck et al., 1984), it could be speculated that DBI mRNA expression in the hypothalamus is negatively regulated by CRF and/or other hormones responsive to the stressful stimuli and that such a regulatory mechanism contributes to social isolation-induced decrease in DBI mRNA expression in the same brain region. To clarify such a feedback mechanism of DBI mRNA expression requires measurement of the hypothalamic DBI mRNA expression at the early stages of social isolation stress exposure.

It is also possible that the decrease in DBI mRNA expression following long-term social isolation produces a reduction of DBI peptide in the hypothalamus and that this decrease may be implicated in pentobarbital sleep disorder in isolated mice. Biochemical evidence has demonstrated that, besides the negative allosteric modulation of the $GABA_A$ receptor via central type BZD receptors, DBI also indirectly affect $GABA_A$ receptor function by acting on glial cell mitochondrial peripheral-type BZD receptors, thereby stimulating the production of neurosteroids that positively or negatively modulate $GABA_A$ receptor function (Costa, 1991; Costa and Guidotti, 1991). Recently Matsumoto et al. (1998) found that long-term social isolation decreased the brain content of allopregnanolone, a positive allosteric modulator of the $GABA_A$ receptor and suggested that down regulation of the $GABA_A$ receptor caused by decrease of allopregnanolone is implicated in the decrease of pentobarbital sleep caused by social isolation stress. Thus, a reduction in the DBI content may contribute to a decrease of neurosteroids with positive allosteric action on the $GABA_A$ receptor, resulting in a decrease of $GABA_A$ receptor function in the socially isolated animals. This idea, however, conflicts with the data obtained from previous behavioral experiments that administration of exogenous DBI or ODN to group-housed animals produced a similar effect to that of social isolation stress; both DBI and ODN decreased the duration of pentobarbital sleep and flumazenil reversed the action of these peptides (Dong et al., 1998). So far, no information is available on whether DBI predominantly enhances neurosteroid(s) with positive, or negative allosteric action on the $GABA_A$ receptor. Thus, to clarify the exact relationship between the changes in DBI mRNA expression and the content of DBI peptide in the hypothalamus following long-term social isolation requires further investigations. Such investigations are in progress in this laboratory.

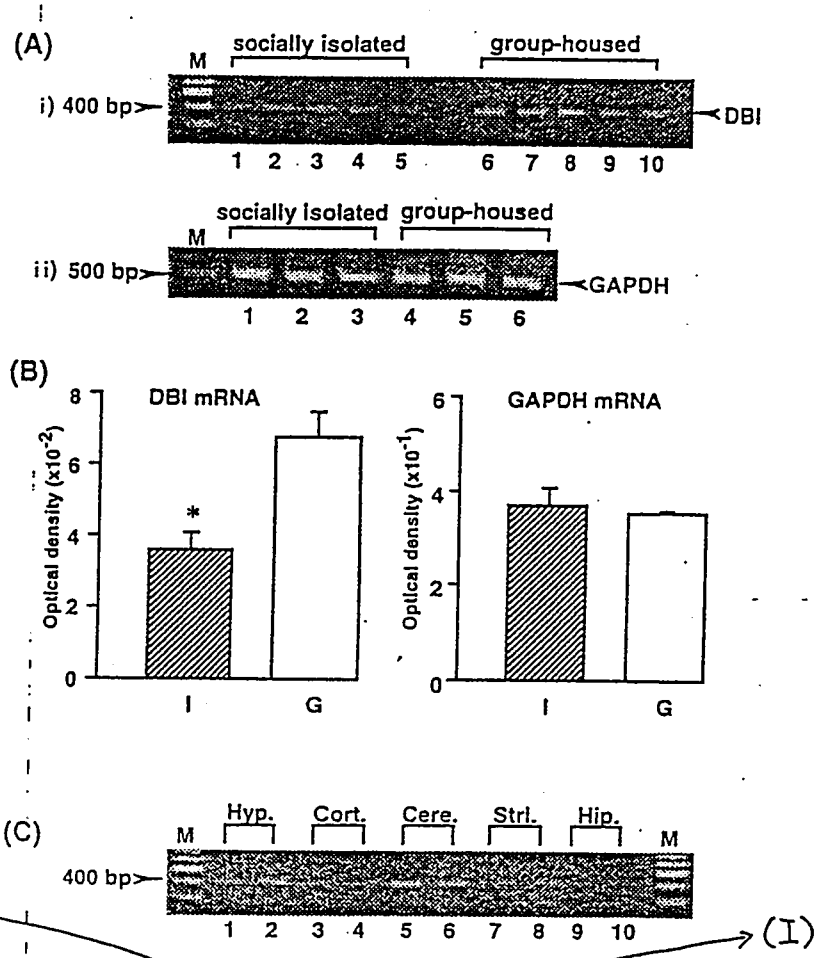

Fig. 3. Social isolation stress-induced changes in the DBI gene level in the brain. Mice were either group-housed or individually isolated for 6 weeks before the start of the experiments. DBI mRNA was extracted from the brains of each animal groups. (A) Digitized image of DBI mRNA (i, lanes 1–5: socially isolated mice; lanes 6–10: group-housed mice) and GAPDH mRNA (ii, lanes 1–3: socially isolated mice; lanes 4–6: group-housed mice) extracted from the hypothalamus region of socially isolated and group-housed mice. (B) Quantification of DBI and GAPDH mRNA signals in the hypothalamus region of group-housed and socially isolated mice. Each data column represents the mean ± S.E.M. ($n = 3–5$). *$P < 0.001$ (Student's t-test). (C) DBI mRNA expressed in the hypothalamus (Hyp.), cortex (Cort.), cerebellum (Cere.), striatum (Stri.), and hippocampus (Hip.) of group-housed (G) and socially isolated mice (I). DBI mRNA extracted from each brain region of socially isolated (lanes 1, 3, 5, 7 and 9) and group-housed mice (lanes 2, 4, 6, 8 and 10) was amplified as described in the text.

In conclusion, we demonstrated for the first time that DBI gene expression in mouse brain, especially in the hypothalamus region, can be suppressed by long-term social isolation stress. This finding provides an evidence that DBI is actually involved in shortened pentobarbital sleep caused by isolation stress in mice. However, according to the general biochemical evidence suggesting that level of gene should parallel that of its transcripted protein, the present results seemed hardly to agree with the findings of our previous behavioral findings. To explain it, we discussed the possible mechanisms underlying this decrease of DBI mRNA expression such as a negative feedback regulation of DBI mRNA expression by DBI peptide, other endogenous substance(s) with more potent activity than DBI, or stress-related hormones. Nevertheless, further investigations are required to test these possibilities.

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