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〔Original Article〕

Induction of Neuroserpin Expression in Rat Frontal Cortex after Chronic Antidepressant Treatment and Electroconvulsive Treatment

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Abstract: Using expressed sequence tag (EST) analysis, we previously identified certain molecular machinery that mediates antidepressant effects. To date, several partial cDNA fragments, termed antidepressant-related genes (ADRGs), have been isolated as ESTs from rat brain. In the present study, we identified two of the ADRGs to be rat neuroserpin. Using real-time quantitative PCR, we demonstrated increased neuroserpin mRNA expression in rat frontal cortex after chronic treatment with several classes of antidepressants, including imipramine, fluoxetine, sertraline, and venlafaxine. Electroconvulsive treatment (ECT), another therapeutic treatment for depression, also increased neuroserpin expression in rat frontal cortex. Neuroserpin is a serine protease inhibitor that is implicated in the regulation of synaptic plasticity, neuronal migration, and axogenesis in the central nervous system. In conclusion, our results support the hypothesis that neuroserpin-mediated plastic changes in frontal cortex may underlie the therapeutic action of antidepressants and ECT.

Key words: Depression, Neuronal plasticity, tPA, Microarray

Depression is one of the major psychiatric diseases. It is characterized by abnormal emotional, cognitive, autonomic, and endocrine functions. Typical antidepressants acutely inhibit monoamine reuptake, resulting in a significant increase in synaptic concentration of monoamines (noradrenaline or serotonin) (Yamada and Higuchi, 2002). With antidepressants, there is a latency period of several weeks before the onset of clinical improvement. Chronic electroconvulsive treatment (ECT) is another therapy that is widely used to alleviate depression, particularly for treating drug-resistant depression. While ECT is effective, its therapeutic mechanism remains unknown. The therapeutic benefits of ECT also have a delayed onset.

For both treatment modalities, this delay could be the result of indirect regulation of neural signal transduction systems or molecular changes in gene transcription. Indeed, antidepressants have been reported to selectively affect specific immediate early genes and transcription factors (see review by Yamada and Higuchi, 2002). Thus, it is reasonable to propose that these molecules may have an important role in the adaptive neural changes that occur following chronic antidepressant treatment. Identification of quantitative changes in gene expression that occur in the brain after chronic antidepressant treatment might yield novel molecular markers that would be useful in diagnosing and treating depression. Using expressed sequence tag (EST) analysis, we and other groups have isolated genes that are differentially expressed in rat brain after chronic antidepressant treatment (Drigues et al, 2003; Wong et al, 1996; Huang et al, 1997; Yamada et al, 1999, 2000, 2001, 2002). To date, we have cloned several partial cDNA fragments as ESTs, which we named antidepressant-related genes (ADRGs).

In the present study, we focused on ADRG116 and ADRG604, both of which were isolated independently and were found to correspond to rat neuroserpin. Neuroserpin is a neural serpin that inhibits the extracellular protease tissue-type plasminogen activator (tPA). Neuroserpin was first identified as an axonally secreted glycoprotein in neuronal cell cultures of chicken dorsal root ganglia (Osterwalder et al, 1996). It is widely expressed in the developing and adult nervous systems and is implicated in the regulation of proteases involved in synaptic plasticity, neuronal migration, and axogenesis (Hastings et al, 1997; Krueger et al, 1997; Schrimpf et al, 1997). Neuroserpin is also implicated in the regulation of emotional behavior, such as anxiety-like responses (Madani et al, 2003). Here, we provide the first report demonstrating that neuroserpin is expressed in the rat frontal cortex and that it is upregulated after chronic treatment with antidepressants and ECT.

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Abbreviations **ADRG**: antidepressant-related gene, **ECT**: electroconvulsive treatment, **EST**: expressed sequence tag, **tPA**: tissue-type plasminogen activator

METHODS

Experimental animals

Male Sprague-Dawley rats (age: 7–10 weeks; Sankyo Labo Service Co., Tokyo, Japan) were housed in a temperature-controlled environment with a 12-hour light/12-hour dark cycle; they had free access to food and water.

Experimental treatments

Rats were randomly assigned to either a control or treatment group. Treatments consisted of either antidepressant drugs or ECT. Six rats were used for antidepressant experiments and 4 rats were used for ECT experiments. We used the following antidepressant drugs for treatment: the tricyclic imipramine (Sigma-Aldrich, Inc., MO, USA), the selective serotonin reuptake inhibitors fluoxetine (The Lilly Laboratories, IN, USA) and sertraline (Pfizer Pharmaceuticals Inc., NY, USA), and the serotonin noradrenaline reuptake inhibitor venlafaxine (Wyeth, PA, USA). Animals in the antidepressant-treatment group received by daily intraperitoneal injection either vehicle, imipramine (10 mg/kg), fluoxetine (5 mg/kg), sertraline (10 mg/kg), or venlafaxine (10 mg/kg) for 1 day or 21 days. We determined the dose for each of the antidepressants according to the previous reports (Nibuya et al, 1996; Pei et al, 2004; Yamada et al, 2001, 2002). All drugs were dissolved in physiological saline containing 1.5% Tween-80. Rats in the ECT group were anesthetized with sevoflurane (Maruishi Pharmaceutical Co., Ltd., Osaka, Japan) before receiving a 90 mA, 0.1 sec electric shock via ear-clip electrodes. Shocks were delivered with a Ugo Basile Model 7801 Unipolar square-wave electroconvulsive stimulation pulse generator (Stoelting Co., IL, USA). Animals received either a single shock (single administration ECT group), or shocks every other day for 14 days (chronic administration ECT group). The control group was treated exactly as the ECT-treatment group, except that electric current was administered.

Animals were killed by decapitation 24 hours after the final antidepressant or ECT treatment; and the brain was quickly removed, dissected, frozen immediately in liquid nitrogen, and stored at −80°C until analysis. All studies using animals were carried out in accordance with animal protocols approved by The Institutional Animal Care and Use Committee of Showa University.

EST analysis

Total RNA from rat frontal cortex was extracted in Isogen reagent (Nippon Gene Co., Ltd., Toyama, Japan) according to the manufacturer's instructions. Isolated total RNA was then dissolved in RNase-free water, and RNA concentration was estimated by UV spectrometry. Total RNA samples were treated with RNase-free DNase I (Nippon Gene Co., Ltd.) for 30 min at 37°C, then were purified by phenol-chloroform extraction. The first-strand cDNA was synthesized with reverse transcriptase (Invitrogen, CA, USA), 1 μM of oligo-dT primer, and 2 μg of total RNA treated with DNase I, then diluted to a final volume of 100 μl. EST analysis was then carried out in the presence of [^{33}P] dATP (Life Science Products, Inc., MA, USA) with an mRNA fingerprinting kit (Clontech, CA, USA) according to the manufacturer's instructions. Radiolabeled PCR products were then analyzed electrophoretically on denaturing 6% polyacrylamide gels. Three individual samples from each treatment group were applied side-by-side and visualized by autoradiography.

Identification of both ADRG116 and ADRG604

ADRG microarray development and fluorescence image analysis was done as described previously (Yamada et al, 2000). Briefly, we amplified each ADRG cDNA insert using vector primers and negative controls, and we spotted in duplicate ten different kinds of housekeeping genes onto glass slides using a GMS417 Arrayer (Affymetrix, Inc., CA, USA). To make the fluorescence-labeled probe for hybridization, poly-A$^+$ RNA was purified from total RNA that was pooled from three independent control or treated groups. Probes were fluorescently tagged by converting 1 μg of poly-A$^+$ RNA from the control and treated samples to cDNA in the presence of either Cy-5- or Cy-3-dUTP, respectively. Hybridization of probes to the microarray was done competitively and in duplicate. The probes were mixed and placed on an array, overlaid with a coverslip, and allowed to hybridize for 16.5 hours at 65°C. After hybridization and washing, each slide was scanned with a GMS418 Array Scanner (Affymetrix, Inc.). Gene expression levels were quantified and analyzed using ImaGene software (Bio-Discovery Ltd. Swansea, UK). Sequence analysis of both ADRG116 and ADRG604 was performed by dideoxy sequencing methods. Homology search and sequence alignment was done using the FASTA search servers at the National Center for Biotechnology Information.

Real-time quantitative PCR

Previously, two cDNAs encoding neuroserpin, rNS-1 (2917 bp) and rNS-2 (1599 bp), were reported (GenBank accession numbers AF193014 and AF193015 respectively; Hill et al, 2000). The principal difference between them was the length of the 3′ untranslated region. We have found that ADRG604 corresponds to rNS-1 (Fig. 1). Therefore, we have analyzed the changes of rNS-1 expression in mRNA level in the present study. PCR primers were designed with Primer Express Software (Applied Biosystems). The primers used for rNS-1 were 5′-CAGCACATTTTCACAGACAGAGATT-3′ and 5′-AGGGCATTGTATTATTAGTGCAGATTAA-3′ (Invitrogen) (black arrows in Fig. 1). The primers we used for amplification of the β-actin reference gene were 5′-TCGCTGACAGGATGCAGAAGG-3′ and 5′-GCCAGGATAGAGCCACCAAT-3′ (Invitrogen). We confirmed the presence of a single PCR product on agarose gel after electrophoresis. In addition PCR fragments were sequenced, and their identity was ascertained (data not shown).

Quantification of rNS-1 expression in rat brain was performed with real-time quantitative PCR and an ABI PRISM 7000 instrument (Applied Biosystems, CA, USA). A quantity of cDNA corresponding to 20 pg of total RNA was amplified by PCR in duplicate. The SYBR® Green PCR Core Reagents Kit (Applied Biosystems) was used for fluorescent detection of cDNA. Real-time quantitative PCR conditions were as follows: 50°C for 2 min then 95°C for 10 min for one cycle, followed by 50 cycles of 95°C for 15 s, 60°C for 1 min. We performed the real-time quantitative PCR amplification of rNS-1 and β-actin at the same time. Then we used the

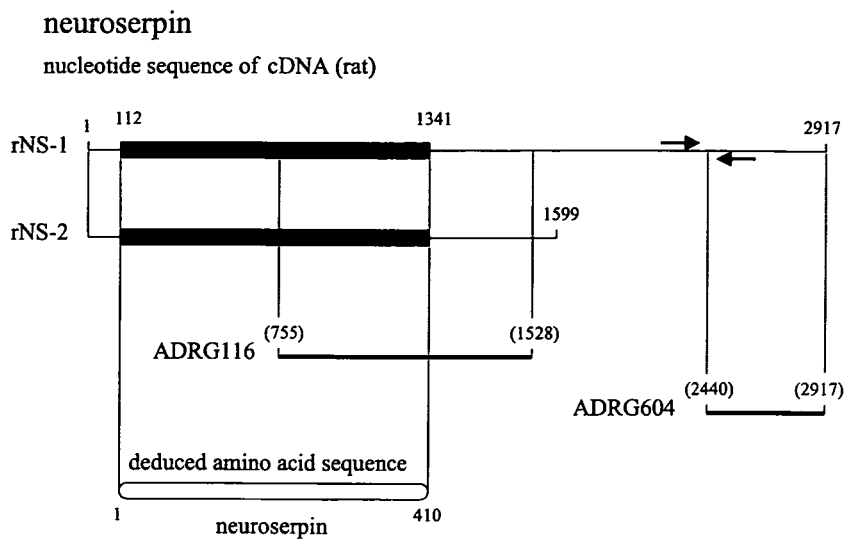

Fig. 1 Structure of the cDNAs encoding rat neuroserpin, rNS-1 and rNS-2 (GenBank accession numbers AF193014 and AF193015 respectively) and their deduced amino acid sequence. Homology analysis of ADRG116 and ADRG604 using DDBJ/EMBL/GenBank databases revealed significant matches to the rat neuroserpin gene. The sizes of the ADRG116 and ADRG604 fragments obtained from EST analysis were 774 bp and 478 bp, respectively. Black arrows represent the oligonucleotide primer pair used for real-time quantitative PCR experiments.

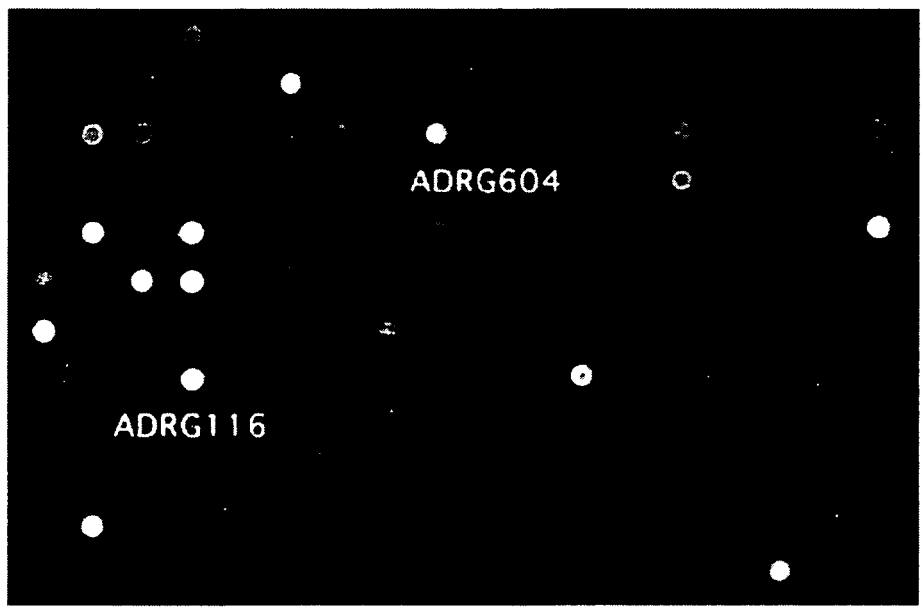

Fig. 2 Image analysis of ADRG microarray after hybridization with fluorescent probes. The pseudo-color image representing hybridization within the control sample (green) and the chronic sertraline-treatment sample (red) are overlapped. As expected, we obtained low background and consistent results in duplicate experiments. The spots within the blue rectangles represent ADRG116 and ADRG604. Interestingly, the fluorescence intensities of the spots representing ADRG116 and ADRG604 increased 3.72 times and 1.67 times, respectively, after chronic sertraline treatment compared to those of the controls.

Table 1 rNS-1 expression in rat brain after chronic antidepressant treatment as assessed by real-time quantitative PCR[a)]

Brain region	Treatment				
	Control	Imipramine	Fluoxetine	Sertraline	Venlafaxine
Frontal cortex	100±5	130±12	126±2*	149±15	137±12*
Hippocampus	100±15	81±8	102±15	74±8	82±6
Hypothalamus	100±4	104±8	120±10	118±15	94±17

a) Total RNA was extracted from rat brain after each treatment and used for real-time quantitative PCR (see Methods; $n=6$). Data represent percentages of control values (means±SEM). Differences were assessed using Student's t-test. *: $P<0.05$ was regarded as significant.

Table 2 rNS-1 expression in rat brain after ECT as assessed by real-time quantitative PCR[a)]

Brain region	Treatment		
	Control	Single administration	Chronic administration
Frontal cortex	100±6	120±16	121±8*
Hippocampus	100±10	85±8	80±4
Hypothalamus	100±12	89±5	89±7

a) Total RNA was extracted from rat brain after each treatment and used for real-time quantitative PCR (see Methods; $n=4$). Data represent percentages of control values (means ± SEM). Differences were assessed using Student's t-test. *: $P<0.05$ was regarded as significant.

Standard Curve Method (User Bulletin, ABI PRISM 7000 Sequence Detection System) for quantification. Briefly, for rNS-1 and β-actin, an absolute standard curve was obtained by plotting the cycle of threshold following PCR amplification of serial dilutions of the control cDNA template. Data are given as percentages of control values (means ± SEM). Differences were assessed using Student's t-test. A value of $P<0.05$ was regarded as statistically significant.

RESULTS

Identification of ADRG116 and ADRG604 as rat neuroserpin

In the present study, we used an ADRG microarray for high-throughput secondary screening to identify genes commonly affected by antidepressants. Fig. 2 shows the pseudocolor image of the ADRG microarray after hybridization with samples obtained from frontal cortex of sertraline-treated rats. As expected, we obtained low background and consistent results in duplicate experiments. After normalization of the signals with both negative and positive controls, several spots of interest on the ADRG microarray showed increased or decreased fluorescence intensities after chronic sertraline treatment. Interestingly, the fluorescence intensities of the spots for both ADRG116 and ADRG604 increased 3.72 times and 1.67 times, respectively, after chronic sertraline treatment when compared to controls (Fig. 2).

Sequence and homology analysis of ADRG116 and ADRG604 (identified by EST analysis to be two independent clones) using DDBJ/EMBL/GenBank databases revealed significant matches to the rat neuroserpin gene (Hill et al, 2000). This finding strongly indicated that neuroserpin was upregulated after chronic sertraline treatment. Fig. 1 shows the structure of the cDNAs encoding rat neuroserpin and the deduced amino acid sequence. The open reading frame spanned from the 112th base to the 1341st base. The deduced amino acid sequence of neuroserpin contained 410 amino acid residues and a putative reactive site loop that binds the active site of target proteases (Osterwalder et al, 1996). The sizes of the ADRG116 and ADRG604 fragments obtained from EST analysis were 774 bp (755–1528th) and 478 bp (2440–2917th), respectively.

Real-time quantitative PCR

The differential expression of rNS-1 in both antidepressant and ECT groups was confirmed by real-time quantitative PCR. As shown in Table 1, chronic administration of imipramine, fluoxetine, sertraline, and venlafaxine increased rNS-1 expression in rat frontal cortex when compared to control samples. On the other hand, single administration of these antidepressants did not affect rNS-1 expression in this region of the brain (data not shown). Interestingly, in the hippocampus and hypothalamus, chronic treatment with these antidepressants did not significantly affect rNS-1 expression (Table 1).

The expression of rNS-1 was also significantly increased in rat frontal cortex after chronic administration of ECT (Table 2). Single administration of ECT also increased rNS-1 expression, but not significantly. Single and chronic administration of ECT did not affect rNS-1 expression in the hippocampus or hypothalamus (Table 2).

DISCUSSION

The serine protease tPA is found not only in the blood where it primarily acts as a thrombolytic enzyme, but also in the central nervous system where it promotes events

associated with synaptic plasticity. Neuroserpin, a serine protease inhibitor (serpin) that reacts preferentially with tPA, is located in regions of the brain where tPA protein is also found, indicating that neuroserpin is a selective inhibitor of tPA in the central nervous system. Growing evidence suggests that tPA participates in many physiological and pathological events in the central nervous system, and that neuroserpin plays an important role as a natural regulator of tPA activity in these processes (Hastings et al, 1997; Yepes and Lawrence, 2004).

In the adult mouse central nervous system, neuroserpin is most strongly expressed in the neocortex, the hippocampal formation, the olfactory bulb, and the amygdala (Krueger et al, 1997). During embryonic development in the mouse, it is expressed in the differentiating fields of most central nervous system regions (Krueger et al, 1997). Moreover, neuroserpin has been reported to regulate neurite outgrowth in nerve growth factor-treated PC12 cells (Parmar et al, 2002). Interestingly, neuroserpin-deficient mice display a selective reduction in locomotor activity in novel environments, an anxiety-like response in the O-maze, and a neophobic response to novel objects (Madani et al, 2003). Neuroserpin-overexpressing mice display reduced center exploration in the open-field test and neophobic responses in a novel object test. These findings suggest that neuroserpin plays some sort of role in the regulation of emotional behavior (Madani et al, 2003).

In the present study, we observed a significant increase in rNS-1 expression in rat frontal cortex after chronic treatment with antidepressants. This altered pattern of rNS-1 expression was also observed in rat frontal cortex after chronic ECT. On the other hand, a single administration of antidepressants failed to induce rNS-1 expression, suggesting that the induction of rNS-1 is due to the long-term therapeutic action of antidepressants. Clinically, a period of several weeks passes before the onset of the therapeutic effect of antidepressants.

As shown in the results, the data obtained from the microarray study was more apparent than that of real-time quantitative PCR. This would be due to the technical differences between hybridization-based analysis and PCR-based quantification. We believe that the real-time quantitative PCR is much more accurate than the microarray analysis.

Although induction of rNS-2 by antidepressants and ECT are yet to be elucidated, our data suggest that neuroserpin may be one of the common functional molecules induced after these treatments. In addition, we need to confirm the induction of neuroserpin at the protein level. We did not use ANOVA for statistical analysis due to the small number of subjects for each treatment. Therefore, we must be careful in interpreting our observations.

The frontal cortex is one of several brain regions involved in the endocrine, emotional, cognitive, and vegetative abnormalities found in depressed patients. Indeed, glucose metabolism, blood flow, and electroencephalographic activity are altered in the frontal cortex of depressed patients (Drevets et al, 1992).

It has also been suggested that the long-term actions of antidepressants may be mediated by changes in neural plasticity (Duman, 2002; McEwen and Olie, 2005; Yamada and Higuchi, 2002). Alterations in certain functional proteins (e.g., protein kinase C and GAP-43) related to neural plasticity have been reported in the brain of depressed suicide victims (Hrdina et al, 1998). Taken together, our results also support the hypothesis that neuroserpin-mediated plastic changes in the frontal cortex may contribute to the therapeutic actions of antidepressants and ECT.

In rat hypothalamus, rNS-1 expression was not affected by chronic antidepressant administration or chronic ECT. Interestingly, in rat hippocampus, rNS-1 expression seemed to have a tendency to decrease with these treatments, although not significantly. The hippocampus and hypothalamus represent other regions of the brain implicated in the pathophysiology of depression (Sheline et al, 1996; Barden, 2004). Although pharmacological actions targeting a single brain region may possibly mediate the therapeutic effects of antidepressants, pharmacological actions targeting multiple brain regions are more likely to contribute to the actual therapeutic effects of antidepressants and ECT. Studies to further characterize the neuronal circuitry of these brain regions will help to elucidate the neuroanatomical substrates of antidepressant effects. We used only one dose for each antidepressant in the present study. rNS-1 expression in hippocampus or hypothalamus could be changed with higher doses.

In this study, a single administration of ECT also induced rNS-1 expression in rat frontal cortex, although not significantly. Clinically, ECT has been considered to be a more rapid and effective treatment for major depression than are antidepressant drugs (Segman et al, 1995). Our results are consistent with the hypothesis that the rapid induction of neuroserpin in frontal cortex underlies the relatively rapid onset of the therapeutic action of ECT. Further characterization of neuroserpin as a functional protein in the central nervous system is needed to test this hypothesis.

In conclusion, we identified neuroserpin as a novel molecular target for antidepressants and ECT. Our findings offer novel insights into the actions of these treatments that may be of both basic and clinical significance.

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Full Paper

Changes in Emotional Behavior of Mice in the Hole-Board Test After Olfactory Bulbectomy

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Abstract. The most consistent behavioral change caused by olfactory bulbectomy (OBX) is a hyperemotional response to novel environmental stimuli. The aim of this study was to characterize the emotional behavior of OBX mice using the hole-board test. After the olfactory bulbs were lesioned, sham and OBX mice were housed in single cages for 14 days. The number of head-dips in the hole-board test in single-housed OBX mice was significantly greater than that in single-housed sham mice. The head-dipping behaviors in single-housed sham and OBX mice were reversed by treatment with diazepam, a typical benzodiazepine anxiolytic. (±)-8-Hydroxy-2-(di-*n*-propylamino) tetraline hydrobromide (8-OH-DPAT), a selective 5-HT_{1A}-receptor agonist that has a non-benzodiazepine anxiolytic-like effect, and (+)-4-[(a*R*)-a-((2*S*,5*R*)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N*,*N*-diethyl benzamide (SNC80), a -opioid-receptor agonist, also significantly reversed the number of head-dips in single-housed sham and OBX mice. In conclusion, we suggest that the single-housed OBX mice showed heightened emotional behavior (e.g., increase in head-dipping behavior) in the hole-board test. In addition, we suggest that the hyperemotional behavior characterized by head-dipping behavior in OBX mice was selectively reversed by benzodiazepine and non-benzodiazepine anxiolytics.

Keywords: olfactory bulbectomy, hole-board test, anxiolytic, opioid receptor

Introduction

A well-established syndrome of behavioral, physiological, neurochemical, and neuroendocrine changes resembling those seen in clinical depression emerges in rodents following bilateral ablation of the olfactory bulbs. Therefore, olfactory bulbectomy (OBX) in rodents has been proposed as a model for chronic psychomotor agitated depression, which also has high predictive validity (1 – 3). The most consistent behavioral change caused by olfactory bulbectomy is a hyperemotional response to novel environmental stimuli such as a brightly lit open field apparatus and handling (4, 5). It has been reported that this hyperemotionality of OBX rats is reduced by the acute administration of anxiolytics such as diazepam and chlordiazepoxide or chronic, but not acute, treatment with antidepressants (4 – 8). Many studies have shown that the hyperemotional response in OBX rats might be related to increases in defensive and anxiety-related behaviors or changes in aversively motivated behavior (see review, ref. 5). While the OBX model has been extensively characterized in rats, fewer studies have been conducted with mice. On the other hand, it has been cautioned that data obtained in rats should not be over-extrapolated to those in mice (2). Apart from the characteristic hyperlocomotion, the OBX mouse also models deficits in active and passive avoidance learning (9 – 12) and in spatial and short memory (13, 14). It was suggested that these deficits might derive from the removal of some normal inhibitory influences regulated by the olfactory

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bulbectomy. However, the detailed changes in the emotional behavior of mice after olfactory bulbectomy are not well known.

The hole-board test, which was first introduced by Boissier and Simon (15), provides a simple method for measuring the response of an animal to an unfamiliar environment. Some advantages of this test are that several behaviors can be readily observed and quantified, which makes possible a comprehensive description of the animal's behavior. The use of the hole-board method as a test for anxiolytics has relied on the hypothesis that the behavior of animals exposed to a novel situation results from competition between an exploratory tendency and a withdrawal tendency (16). Thus, according to these hypotheses, a high level of anxiety results in decreased head-dipping behavior, and a low level of anxiety manifests as increased head-dipping behavior: this is the basic assumption that underlies the hole-board paradigm (16 – 20). However, contradictory results have been obtained by several authors (21 – 26). Bilkei-Gorzo and Gyertyan (24) reported that brighter lighting, which was highly aversive environment, increased the head-dipping behavior of rats. Hoshino et al. (25) also reported that the higher frequency of head-dipping behavior was observed in the rats with chronic electrolytic lesion of the raphe nucleus, which was produced higher anxiety levels in rats. Tsuji et al. (26) also reported that the mouse exposure to chronic mild stress for 1 week produced a significant increase in the head-dipping behavior. Indeed, it has been recognized that either increased or decreased head-dipping behavior on the hole-board was able to estimate objectively various emotional states of the animal. However, little is known about the influences in the head-dip behavior of mice following bilateral ablation of the olfactory bulbs.

Recent studies with mice lacking -opioid receptors (27) or preproenkephalin-derived peptides (28, 29) revealed that both types of knockout mice showed higher anxiety levels. We also found that -opioid-receptor antagonists produced anxiety-related behaviors (30). Thus, there is substantial evidence to show that the absence of opioidergic systems modulated by -opioid receptors leads to enhanced anxiety-related behaviors in mice. On the other hand, it has been reported that preproenkephalin mRNA and met-enkephalin-like immunoreactivity levels are increased in the several brain areas in OBX rats (31, 32). In addition, we reported that SNC80, selective -opioid-receptor agonists, might have antidepressant and anxiolytic-like effects using the forced swiming test, the elevated plus maze test, and conditioned fear stress test (33). Therefore, researchers have guessed that -opioid-receptor agonists are as effective in OBX-induced abnormal behaviors as anxiolytics.

The primary aim of this study was to characterize the emotional behavior of mice after olfactory bulbectomy using the hole-board test. Furthermore, we also investigated the effects of housing (single or group housing) on the exploratory behavior of mice after olfactory bulbectomy in the hole-board test, since socially isolated animals are known to exhibit clearly anxiety-like behavior in the elevated plus maze and the open field test (34, 35). In addition to the evaluation of exploratory behavior, we also examined the effects of benzodiazepine, non-benzodiazepine anxiolytics, or -opioid-receptor agonists on the changes in the emotional behavior of OBX mice on the hole-board apparatus.

Materials and Methods

Animals

Experiments were conducted with 4-week-old male ICR mice (Tokyo Laboratory Animals Science, Tokyo), weighing 18 – 23 g. They had free access to food and water in an animal room that was maintained at 24 ± 1°C with a 12-h light-dark cycle. This study was carried out in accordance with the Declaration of Helsinki and the guide for the use of laboratory animals of Hoshi University, which is accredited by the Ministry of Education, Culture, Sports, Science, and Technology.

OBX

In the present study, we subjected 4-week-old mice to OBX and the behavioral test was performed 2 weeks after surgery, since in general the hole-board test is used in 6-week-old mice (17, 19, 20). Mice were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) diluted in 0.9% saline. The skull covering the bulbs was exposed by skin incision and a burr hole was drilled (2.0 mm prior to the bregma, 1 mm lateral to the midline), through which both olfactory bulbs were removed by suction. Sham operations were performed in the same way, but with the skull and bulbs left intact. When the behavioral experiments were complete, mice were decapitated, and the results of bulbectomy were inspected visually. Data obtained from animals with incomplete removal of the bulbs or with frontal cortex damage were discarded. Mice were either single- or group-housed for 14 days. They were handled daily by the experimenter during this period. After the olfactory bulbs were lesioned, the single-housed sham and OBX mice were immediately housed in individual Plexiglas cages (15 × 10 × 12.5 cm). Similarly, after OBX, group-housed sham and OBX mice were immediately housed in groups of 10 in Plexiglas cages (30 × 20 ×

12.5 cm).

The mice were rendered anosmic by infusion of 2.5 μl of a 0.3 M $ZnSO_4$ solution into each nasal cavity (36). The mice were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and positioned in a supine posture with the hind limbs slightly elevated to prevent $ZnSO_4$ from entering the trachea. Animals were housed in a group for 5 days to recover before testing. Anosmia in $ZnSO_4$-treated mice was assessed by measuring the time to approach a novel odor after all behavioral testing was completed. A small cloth soaked in vanilla extract was presented at the top-back corner of the home cage, while the experimenter observed from the front. For each animal, the latency to sniff in the corner containing the novel object was recorded over two testing sessions, each with a maximum duration of 90 s. Two sessions were used to help control for possible visual cues from a cagemate.

Hole-board test

The results in the hole-board test were determined automatically as described previously (37). The hole-board apparatus was made of a gray wooden box (50 × 50 × 50 cm) with four holes 3 cm in diameter equally spaced in the floor and placed in indirect light (40 lx). An infrared beam sensor was installed on the wall to detect the numbers of rearing and head-dipping behaviors, and the latency to the first head-dips. Other behavioral parameters, such as the locus and distance of movement (total locomotor activity (cm)) of mice, were recorded by an overhead color CCD camera. The heads of the mice were painted yellow and the color CCD camera followed the center of gravity. Data from the CCD camera were collected through a custom-designed interface (CAT-10; Muromachi Kikai, Tokyo) as a reflection signal. Head-dipping behaviors were double-checked via an infrared beam sensor and an overhead color CCD camera. All of the data were analyzed and stored in a personal computer using analytical software (Comp ACT HBS, Muromachi Kikai).

After the 14-day post-surgical period, the emotional response in the hole-board test was measured. The drugs were administered 30 min before testing. For the hole-board experiments, each animal was placed in the center of the hole-board and allowed to freely explore the apparatus for 5 min. Total locomotor activity, numbers of rearing and head-dipping behaviors, and the latency to the first head-dip were recorded automatically. Each mouse was used only once. The floor in the hole-board apparatus was cleaned with a paper towel after each trial. Experiments were performed between 13:00 and 17:00.

In experiment 1 (Fig. 1), we examined the effect of housing on the exploratory behavior of mice in the hole-board test. The groups were as follows: naive /group (n = 11), naive/single (n = 11), sham/group (n = 12), sham/single (n = 14), OBX/group (n = 11), OBX/single (n = 14). In experiment 2 (Fig. 2), single-housed naive, sham, and OBX mice were injected with diazepam (s.c.). The groups were as follows: naive /vehicle (n = 10); naive/diazepam, 0.5 mg/kg (n = 8); naive/diazepam, 1.0 mg/kg (n = 8); sham/vehicle (n = 12); sham/diazepam, 0.5 mg/kg (n = 11); sham /diazepam, 1.0 mg/kg (n = 11); OBX/vehicle (n = 13); OBX/diazepam, 0.5 mg/kg (n = 11); OBX/diazepam, 1.0 mg/kg (n = 8). In experiment 3 (Fig. 3), single-housed sham and OBX mice were injected with 8-OH-DPAT (s.c.). The groups were as follows: sham/saline (n = 8); sham/8-OH-DPAT, 0.03 mg/kg (n = 8); sham /8-OH-DPAT, 0.1 mg/kg (n = 8); OBX/saline (n = 11); OBX/8-OH-DPAT, 0.03 mg/kg (n = 11); OBX/8-OH-DPAT, 0.1 mg/kg (n = 11). In experiment 4 (Fig. 4), single-housed sham and OBX mice were injected with SNC80 (s.c.). The groups were as follows: sham/saline (n = 9); sham/SNC80, 1.0 mg/kg (n = 8); sham/SNC80, 3.0 mg/kg (n = 8); OBX/saline (n = 10); OBX/SNC80, 1.0 mg/kg (n = 11); OBX/SNC80, 3.0 mg/kg (n = 11).

Data analyses

The data are expressed as means ± S.E.M. The statistical significance of differences in behavioral data between each group was assessed with two-way analysis of variance (ANOVA), and post hoc individual group comparisons were made with the Tukey-Kramer test. Analyses were made using StatView 5.0 statistical software (SAS Institute, Inc., Cary, NC, USA). *P*-values of less than 0.05 were considered significant.

Drugs

The drugs used in the present study were diazepam (Cercine®; Takeda Pharmaceutical Co., Ltd., Osaka), (±)-8-hydroxy-2-(di-*n*-propylamino) tetraline hydrobromide (8-OH-DPAT; Sigma Chemical Co., St. Louis, MO, USA), and (+)-4-[(a*R*)-a-((2*S*,5*R*)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N*,*N*-diethyl benzamide (SNC80; synthesized by Toray Industries, Inc., Kanagawa). Diazepam (Cercine®), which was an injection of the drug dissolved in 40% benzyl alcohol, 10% ethanol, 1.5% propylene glycol, and 42.8 mg/ml benzoic acid, was diluted with 0.1% Tween 80 physiological saline just before an experiment start. 8-OH-DPAT and SNC80 were dissolved in saline. Each drug was injected 30 min before the hole-board test.

Results

Effects of group- and single-housing on the exploratory behavior of mice in the hole-board test

Figure 1A shows the effects of group- and single-housing on the head-dip counts of mice in the hole-board test. Two-way (housing and surgery) factorial ANOVA revealed a significant housing × surgery interaction [housing × surgery interaction: $F(2, 67) = 6.645$, $P<0.05$]. Post-hoc test analysis showed that although the number of head-dips in single-housed naive and sham mice was less than that in group-housed naive and sham mice, there were no significant differences. In addition, there were no significant differences in head-dip counts between group- and single-housed OBX mice. However, the number of head-dips in single-housed OBX mice was significantly greater than that in single-housed sham mice.

Figure 1B shows the effects of group- and single-housing on the head-dip latency of mice in the hole-board test. Two-way factorial ANOVA revealed significant main effects for housing and surgery, but not housing × surgery interaction [housing: $F(1, 67) = 15.472$, $P<0.05$, surgery: $F(2, 67) = 9.795$, $P<0.05$] (Fig. 1B). These analyses revealed that single-housing significantly increased the head-dip latency in all three surgery conditions.

Figure 1C shows the effects of group- and single-housing on the locomotor activity of mice in the hole-board test. Two-way factorial ANOVA revealed a significant main effect for housing, but not housing × surgery interaction [housing: $F(1, 67) = 16.631$, $P<0.05$] (Fig. 1C). As shown in Fig. 1D, a similar effect was found in rearing counts [housing: $F(1, 67) = 13.278$, $P<0.05$]. These analyses revealed that single-housing significantly increased the locomotor activity and rearing counts in all three surgery conditions (Fig. 1: C and D).

Effects of diazepam on the exploratory behavior of single-housed sham and OBX mice in the hole-board test

Figure 2A shows the effects of diazepam on the head-dip counts of single-housed naive, sham, and OBX mice in the hole-board test. Two-way factorial ANOVA revealed a significant main effect for drug × surgery interaction [drug × surgery interaction: $F(4, 79) = 9.228$, $P<0.05$] (Fig. 2A). Post-hoc test analysis showed that in single-housed naive mice, diazepam (1.0 mg/kg, s.c.)

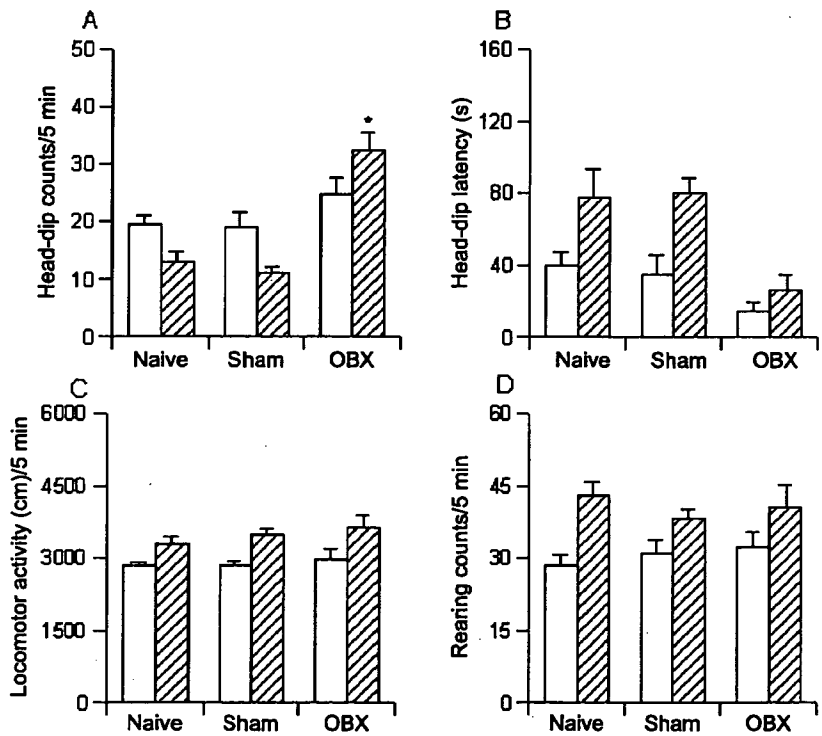

Fig. 1. Effects of group- and single-housing on the exploratory behavior of mice in the hole-board test. Open column represents respective group-housed mice. Closed column represents respective single-housed mice. Changes in the emotional state of mice were evaluated in terms of changes in exploratory activity, that is, number of head-dips (A), latency to the first head-dip (B), total locomotor activity (C), and number of rearing behaviors (D). Saline was injected 30 min prior to the measurement of exploratory behavior. Each column represents the mean with S.E.M. *$P<0.05$ *vs* respective single-housed sham mice.

produced a significant increase in head-dip counts and that diazepam (0.5, 1.0 mg/kg, s.c.) also produced significant increases in head-dip counts in single-housed sham mice. That is, it was indicated that the decrease in head-dipping counts in single-housed naive and sham mice was suppressed by treatment with diazepam. On the other hand, in single-housed OBX mice, diazepam (0.5, 0.1 mg/kg, s.c.) produced a dose-dependent decrease in head-dip counts, and this difference was statistically significant at 0.5 and 1 mg/kg.

Figure 2B showed the effects of diazepam on the head-dip latency of single-housed naive, sham and OBX mice in the hole-board test. Two-way factorial ANOVA revealed a significant main effect for drug × surgery interaction [drug × surgery interaction: $F(4, 79) = 2.998$, $P<0.05$] (Fig. 2B). Post-hoc test analysis showed that diazepam (0.5 mg/kg, s.c.) produced a significant decrease in head-dip counts in single-housed sham mice.

Figure 2C shows the effects of diazepam on the locomotor activity of single-housed naive, sham, and OBX mice in the hole-board test. Two-way factorial ANOVA revealed a significant main effect for drug × surgery interaction [drug × surgery interaction: $F(4, 79) = 3.419$, $P<0.05$] (Fig. 2C). Post-hoc test analysis showed that diazepam (1.0 mg/kg, s.c.) produced a significant increase in locomotor activity in single-housed sham mice. On the other hand, the administration of diazepam had no significant effect on the locomotor activity of single-housed naive and OBX mice.

Figure 2D shows the effects of diazepam on the rearing counts of single-housed naive, sham, and OBX mice in the hole-board test. The rearing counts in single-housed naive, sham, and OBX mice were not significantly affected by treatment with diazepam at doses of 0.5 and 1 mg/kg compared to the respective saline-treated single-housed mice (Fig. 2D).

Effects of 8-OH-DPAT on the exploratory behavior of single-housed sham and OBX mice in the hole-board test

Figure 3A shows the effects of 8-OH-DPAT on the head-dip counts of single-housed sham and OBX mice in the hole-board test. Two-way factorial ANOVA revealed a significant main effect for drug × surgery interaction [drug × surgery interaction: $F(2, 52) = 13.012$, $P<0.05$] (Fig. 3A). Post-hoc test analysis showed that although the effect was not significant, 8-OH-DPAT produced an increase in head-dip counts in single-housed sham mice. That is, it was indicated that

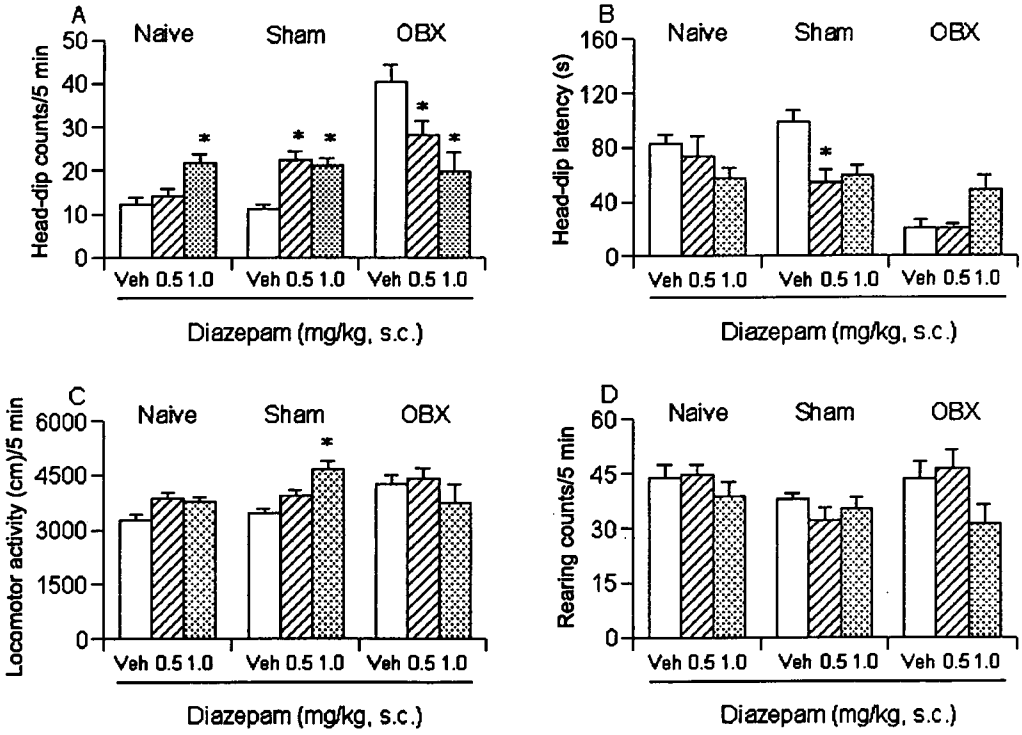

Fig. 2. Effects of diazepam on exploratory behavior of single-housed sham and OBX mice in the hole-board test. Changes in the emotional state of mice were evaluated in terms of changes in exploratory activity, that is, number of head-dips (A), latency to the first head-dip (B), total locomotor activity (C), and number of rearing behaviors (D). Diazepam (0.5, 1.0 mg/kg, s.c.) or saline was injected 30 min prior to the measurement of exploratory behavior. Single-housed sham and OBX mice were immediately housed in a single cage for 14 days after the operation. Each column represents the mean with S.E.M. *$P<0.05$ *vs* respective saline-treated mice.

the decrease in head-dipping counts in single-housed sham mice was suppressed by treatment with 8-OH-DPAT. On the other hand, in single-housed OBX mice, 8-DPAT produced a dose-dependent decrease in head-dip counts, and this difference was statistically significant at 0.03 and 0.1 mg/kg.

Figure 3B shows the effects of 8-OH-DPAT on the head-dip latency of single-housed sham and OBX mice in the hole-board test. Two-way factorial ANOVA revealed a significant main effect for drug × surgery interaction [drug × surgery interaction: $F(2, 52) = 5.355$, $P<0.05$] (Fig. 3B). Post-hoc test analysis showed that 8-OH-DPAT (0.1 mg/kg, s.c.) produced a significant decrease in head-dip counts in the single-housed sham mice. On the other hand, although the effect was not significant, the administration of 8-OH-DPAT increased the head-dip latency of single-housed OBX mice.

Figure 3C shows the effects of 8-OH-DPAT on the locomotor activity of single-housed sham and OBX mice in the hole-board test. Two-way factorial ANOVA revealed a significant main effect for drug × surgery interaction [drug × surgery interaction: $F(2, 52) = 3.983$, $P<0.05$] (Fig. 3C). However, post-hoc test analysis showed that the administration of 8-OH-DPAT had no significant effect on the locomotor activity of single-housed sham and OBX mice.

Figure 3D shows the effects of 8-OH-DPAT on the rearing counts of single-housed sham and OBX mice in the hole-board test. Two-way factorial ANOVA revealed a significant main effect for surgery [surgery: $F(1, 52) = 12.596$, $P<0.05$] (Fig. 3D). These analyses revealed that 8-OH-DPAT did not produce any significant effects in rearing counts in single-housed sham and OBX mice.

Effects of SNC80 on the exploratory behavior of single-housed sham and OBX mice in the hole-board test

Figure 4A shows the effects of SNC80 on the head-dip counts of single-housed sham and OBX mice in the hole-board test. Two-way factorial ANOVA revealed a significant main effect for drug × surgery interaction [drug × surgery interaction: $F(1, 51) = 14.760$, $P<0.05$] (Fig. 4A). Post-hoc test analysis showed that in single-housed sham mice, SNC80 produced, but not significant, increase in head-dip counts. On the other hand, in single-housed OBX mice, SNC80 (1.0 and 3.0 mg/kg)

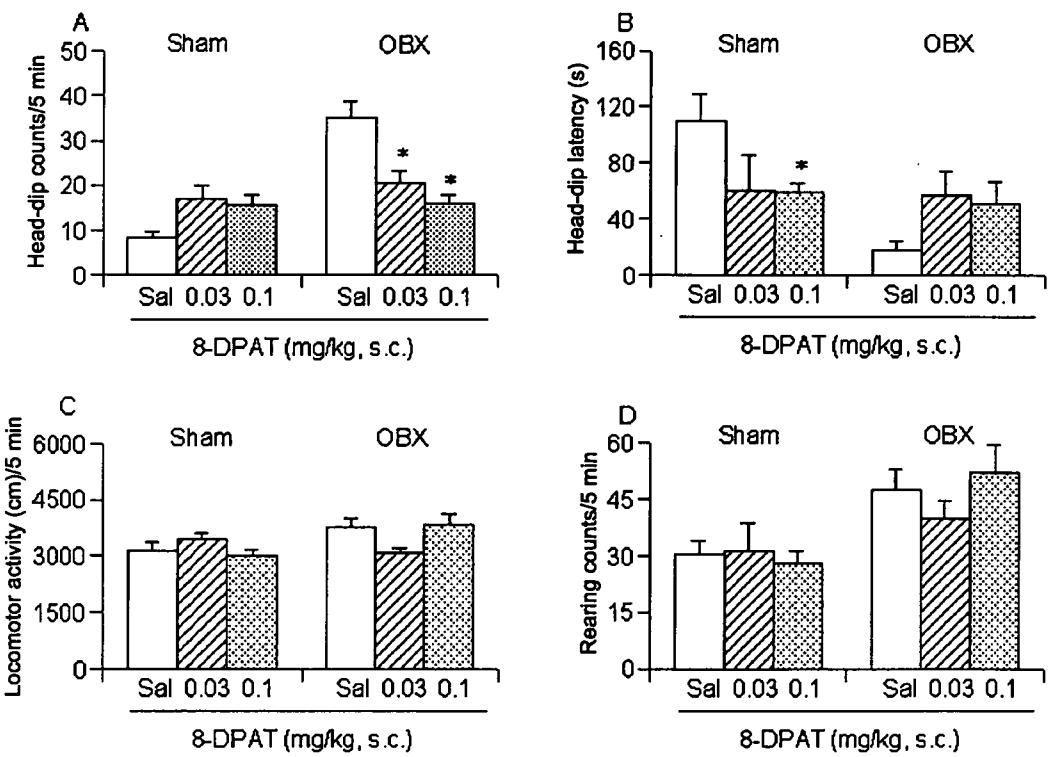

Fig. 3. Effects of 8-OH-DPAT on exploratory behavior of single-housed sham and OBX mice in the hole-board test. Changes in the emotional state of mice were evaluated in terms of changes in exploratory activity, that is, number of head-dips (A), latency to the first head-dip (B), total locomotor activity (C), and number of rearing behaviors (D). 8-OH-DPAT (0.03, 0.1 mg/kg, s.c.) or saline was injected 30 min prior to the measurement of exploratory behavior. Single-housed sham and OBX mice were immediately housed in a single cage for 14 days after the operation. Each column represents the mean with S.E.M. *$P<0.05$ *vs* respective saline-treated mice.

produced a significant decrease in head-dip counts.

Figure 4B shows the effects of SNC80 on the head-dip latency of single-housed sham and OBX mice in the hole-board test. Two-way factorial ANOVA revealed a significant main effect for drug × surgery interaction [drug × surgery interaction: $F(2, 51) = 7.608$, $P<0.05$] (Fig. 4B). Post-hoc test analysis showed that SNC80 (1.0 mg/kg, s.c.) produced a significant decrease in head-dip latency in single-housed sham mice. On the other hand, in single-housed OBX mice, SNC80 produced, but not significant, increase in head-dip latency.

Figures 4C and 4D show the effects of SNC80 on the locomotor activity and rearing counts of single-housed sham and OBX mice in the hole-board test. Treatment with SNC80 had no significant effects for drug, surgery or drug × surgery interaction (Fig. 4: C and D).

Effects of anosmia on the exploratory behavior of $ZnSO_4$-treated mice in the hole-board test

The extent of olfactory impairment was assessed by measuring the latency to sniff a novel odor (vanilla or almond extract) presented over the home cage. $ZnSO_4$-treated mice did not (0 of 6) sniff the odor within the allotted 90 s for both sessions. In contrast, all of the vehicle-treated mice (n = 6) sniffed the novel odor within both 90-s sessions. There was no significant difference in locomotor activity, numbers of head-dipping, and rearing behaviors and latency to the first head-dips between vehicle- and $ZnSO_4$-treated anosmic mice (Table 1).

Discussion

We examined the effects of olfactory bulbectomy on the emotional behavior of mice using an automatic hole-board apparatus. The number of head-dips in single-housed naive mice was significantly less than that in group-housed naive mice. Sham-operated mice showed similar head-dipping behavior. Previously, it has been reported that decreased head-dipping behavior might reflect an anxiety-like behavior in mice (17, 20, 37). Furthermore, social isolation has been proposed as a model for anxiety, since socially isolated animals are known to exhibit anxiety-like behavior in the elevated plus maze and the open field (34, 35). In the present study, the decrease in head-dipping counts in single-housed naive and sham mice were suppressed by treatment with diazepam, a typical benzodiazepine

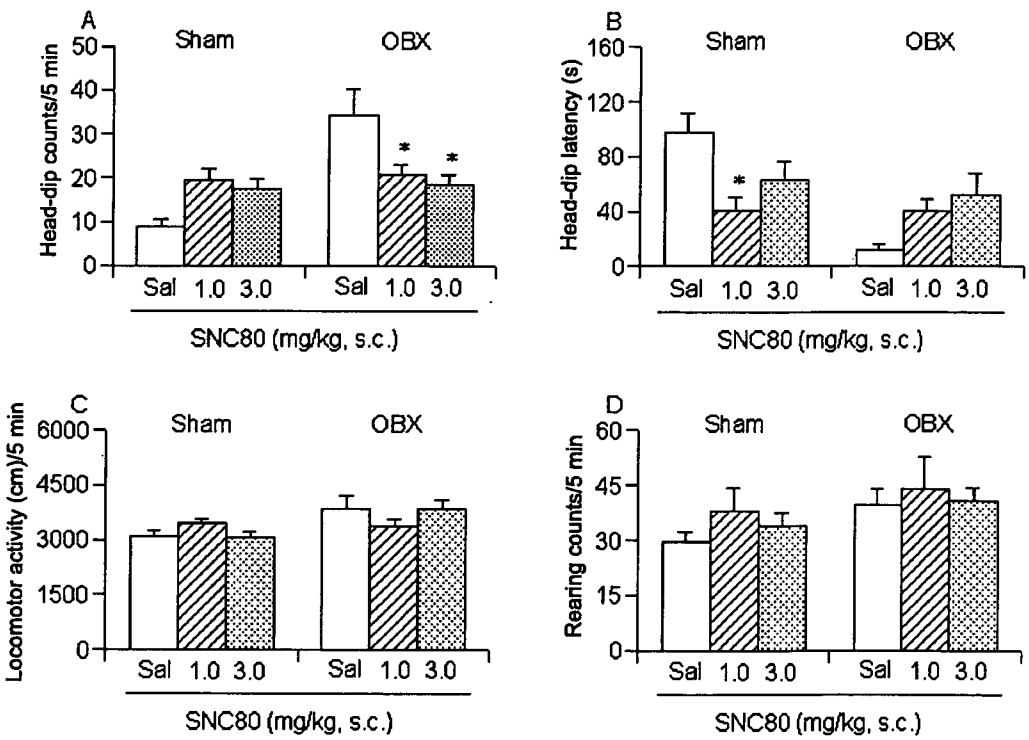

Fig. 4. Effects of SNC80 on exploratory behavior of single-housed sham and OBX mice in the hole-board test. Changes in the emotional state of mice were evaluated in terms of changes in exploratory activity, that is, number of head-dips (A), latency to the first head-dip (B), total locomotor activity (C), and number of rearing behaviors (D). SNC80 (1.0, 3.0 mg/kg, s.c.) or saline was injected 30 min prior to the measurement of exploratory behavior. After the olfactory bulbs were lesioned, single-housed OBX mice were immediately housed in a single cage for 14 days. Each column represents the mean with S.E.M. *$P<0.05$ *vs* saline-treated mice.

Table 1. Effects of anosmia on the exploratory behavior of $ZnSO_4$-treated mice in the hole-board test

Treatment	Locomotor activity (cm)	Rearing counts	Head-dips	
			Counts	Latency (s)
Vehicle-treated mice	1876.2 ± 152.5	41.5 ± 8.3	21.3 ± 5.2	29.4 ± 13.2
$ZnSO_4$-treated mice	2341.7 ± 255.6	44.4 ± 5.2	28.3 ± 4.7	18.7 ± 9.06

The mice were rendered anosmic by infusion 2.5 μl of a 0.3 M $ZnSO_4$ solution into each nasal cavity. Anosmia in $ZnSO_4$-treated mice was assessed by measuring the time to approach a novel odor after hole-boad testing was completed. Each animal was placed in the center of the hole-board and allowed to freely explore the apparatus for 5 min. Each value represents the mean with S.E.M.

anxiolytic, and 8-OH-DPAT, a selective 5-HT_{1A}-receptor agonist, which was known as the non-benzodiazepine anxiolytics (38), without any significant effect on locomotor activity or rearing counts. Previously, it was reported that the decreases in head-dipping behavior in mice produced by stress stimuli could be reversed to the pretreatment levels with diazepam and/or 8-OH-DPAT in the hole-board test (17). Thus, it was suggested that single-housed sham and OBX mice showed heightened emotional behavior and these behaviors could be completely recovered with an anxiolytic drug. In addition, the decrease in head-dipping behavior of single-housed sham mice might reflect the anxiety-like behavior, which is due to social isolation stress. On the other hand, single-, but not group-, housed mice after olfactory bulbectomy showed a significant increase in head-dip counts compared with single-housed sham mice. Furthermore, both benzodiazepine (diazepam) and a non-benzodiazepine anxiolytic (8-OH-DPAT) similarly reversed not only the decrease in head-dipping behavior in sham mice, but also the increase in the head-dipping behavior in OBX mice. Thus, it was indicated that single-housed OBX mice showed heightened emotional behavior (e.g., increase in head-dipping behavior), which could be reversed by benzodiazepine and non-benzodiazepine anxiolytics, in the hole-board test.

In addition, we demonstrated that the changed emotional behavior observed in OBX mice did not result from anosmi since $ZnSO_4$-treated mice did not show a significant increase in head-dipping behavior, locomotor activity, or rearing counts. These results supported our speculation that single-housed mice after olfactory bulbectomy exhibit various significant behavioral changes in the hole-board test. However, it was reported that $ZnSO_4$-treated rats exhibited a significant increase in initial locomotor activity in the open field test, just like OBX rats (36). Thus, it is possible that anosmia may somehow be related to the changed emotional behavior in OBX mice. Further studies are necessary before this possibility can be established with greater certainly.

One of the most consistent findings in the OBX model is an increase in locomotor activity. Bulbectomy has consistently been shown to produce an increase in exploratory behavior, such as locomotor activity and rearing counts in a novel environment such as the open field in several studies (4, 5). However, the present study showed that locomotor activity and rearing counts in all surgery conditions were significantly increased by single-housing. In addition, locomotor activity and rearing counts in single-housed OBX mice were not significantly affected by pretreatment with diazepam, 8-OH-DPAT, or SNC80, in contrast to the results regarding head-dipping behavior. Previously, it was reported that the increased locomotor activity in OBX rats is significantly attenuated by chronic, but not acute, treatment with antidepressants (36, 39). Therefore, chronic treatment with antidepressants may be required to attenuate the increased locomotor activity and rearing counts in OBX mice. OBX-induced locomotor activity and rearing counts observed in the hole-board test might be mediated by distinct neuronal mechanisms, different from head-dipping behavior.

SNC80, a -opioid-receptor agonist, significantly increased the number of head-dips in single-housed sham mice and significantly decreased the number of head-dips in OBX mice. These results support our previous suggestions that -opioid-receptor agonists might have antidepressant and/or anxiolytic-like effects (33). Thus, we proposed that the activation of -opioid receptors might normalize the olfactory bulbectomy-induced emotional abnormality in mice as well as benzodiazepine and 5-HT_{1A}-receptor agonist. In addition, it was suggested that opioid receptors may, at least in part, contribute to in the appearance of OBX-induced emotional abnormality in mice.

Bilkei-Gorzo and Gyertyan (24) reported that brighter lighting (highly aversive environment) increased the head-dipping behavior of rats compared with subdued lighting (moderately aversive environment). They suggested that when the anxiety level of the animals is

too high, fear may result in intense escape attempts manifested by head-dipping behavior, since it is assumed that increasing the level of illumination elevates the anxiety state of the animals. Furthermore, they also reported that the anxiolytic chlordiazepoxide significantly inhibited head-dipping behavior in a highly aversive environment (24). These results resemble our present results. Thus, diazepam reduced the increased head-dip counts in single-housed OBX mice to the same levels as in group-housed sham mice. In contrast, diazepam also elevated the decrease in head-dip counts in single-housed sham mice to the same levels as in group-housed sham mice. It is quite likely that single-housing as well as the OBX procedure might make mice highly stressed and/or anxious. Based on these results, we hypothesize that in a very aversive environment, a hole may represent a means of escape from a fearful environment rather than an explorable place for OBX mice. A further increase in anxiety may lead to an active escape reaction that can reach a state of panic and/or impulsive-like activity. However, further studies are necessary before this possibility can be established with greater certainty.

The present study extends the characterization of the OBX-induced behavioral syndrome in mice using the hole-board test. The behavioral changes described here with respect to the exploratory behaviors such as head-dip behavior are robust and can be achieved by relatively simple test procedures. With the increasing need to test transgenic mice for the presence of abnormal (depressive and/or anxiety-like) behaviors, information about features other than open field hyperactivity should help us to evaluate whether or not a transgenic mouse develops OBX syndrome since hyperlocomotion alone is a common feature in mutant mice (2). These tests and parameters presented here as markers for valid prediction might be a useful procedure in the behavioral characterization of the transgenic mice.

In conclusion, we suggest that the single-housed OBX mice showed heightened emotional behavior (e.g., increase in head-dipping behavior and exploratory activity) in the hole-board test. In addition, we suggest that the hyperemotional behavior characterized by head-dipping behavior in OBX mice was selectively reversed by benzodiazepine and non-benzodiazepine anxiolytics.

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Mapping translational research in the age of theragnostics: from molecular markers to personalized drug therapy

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Translational research is frequently used in the bioscience literature to refer to the translation of basic science into practical applications at the point of patient care. With the introduction of theragnostics, a new medical subspecialty that fuses therapeutics and diagnostic medicine with the goal of providing individualized pharmacotherapy, we suggest that the focus of translational research is shifting. We identify two bottlenecks or gaps in translational research for theragnostics: GAP1 translation from basic science to first-in-human proof-of-concept; and GAP2 translation from clinical proof-of-concept to development of evidence-based personalized treatment guidelines. GAP1 translational research in theragnostics is usually performed in traditional craft-based studies with small sample sizes and led by independent academic or industry researchers. In contrast, GAP2 translational investigations typically rely on large research consortiums and population-based biobanks that couple biomarker information with longitudinal 'real-life' observational data on a broad range of pharmacological phenotypes. Despite an abundance of research on the use of biobanks in disease gene discovery, there has been little conceptual work on whether and to what extent population biobanks can be utilized for translating genomics discoveries to practical treatment guidelines for theragnostic tests.

Keywords: biobanks, bioethics, biomarkers, General Clinical Research Center, theragnostics, personalized medicine, translational clinical research

future medicine

For biomedicine to improve human health, scientific discoveries must be 'translated' into applications at the point of patient care [101]. These applications can be information generating (for example, genetic tests that aid in prediction of disease risk or the individualization of drug therapy) or therapeutic (for example, new drug therapies and medical devices). Research that works between or at the interface of these two poles, that is molecular/preclinical investigations and practical applications in the clinic, is often referred to as 'translational research'.

As an applied science, translational research has a prominent focus on clinically-relevant product development. In the present age of knowledge-based economies [1,2], translational research is increasingly visible and highly sought after by academics, research funding agencies and pharmaceutical or biotechnology industries. However, despite its frequent use in the scientific literature there has been little conceptual work that maps out the process of translational research. For example, is such research a multistage process with several qualitatively different subcomponents? And what does translational research contribute in the context of recent trends towards developing personalized drug therapies? Furthermore, we suggest that translational research is currently being reshaped by the introduction of theragnostics, a term denoting the fusion of therapeutics and diagnostics [3].

Theragnostics indicates a fundamental transformation in pharmaceutical research and medical therapeutics, that is, a move towards codevelopment, and by extension, coprescription of diagnostic tests and drugs to individualize treatment regimens. Unlike routine clinical chemistry (for example., plasma electrolyte measurements) or technology-driven biomarker approaches (for example., genomics), theragnostics does not focus on a single technology platform or marker set, such as blood biochemistry or genetic polymorphisms. Instead, theragnostics relies on an integration of technologies for gathering information from different levels of the biological hierarchy. Thus, a theragnostic approach might include not only pharmacogenomic tests [4] to identify the hereditary basis for individual or population variability in drug effects (whether based on genotype or gene expression), but also include proteomic [5] and metabolomic [6] tests to discern, respectively, the cellular proteins and metabolites formed and degraded under genetic or (patho)physiological influences (Figure 1). For example, trastuzumab (Herceptin®) is a monoclonal antibody directed at the human epidermal growth factor receptor 2 (*HER2*) for use in patients with breast cancer who are *HER2*-positive. Trastuzumab is widely claimed as one of the first generation of personalized medicines, because the drug is prescribed together with a theragnostic test to detect *HER2*

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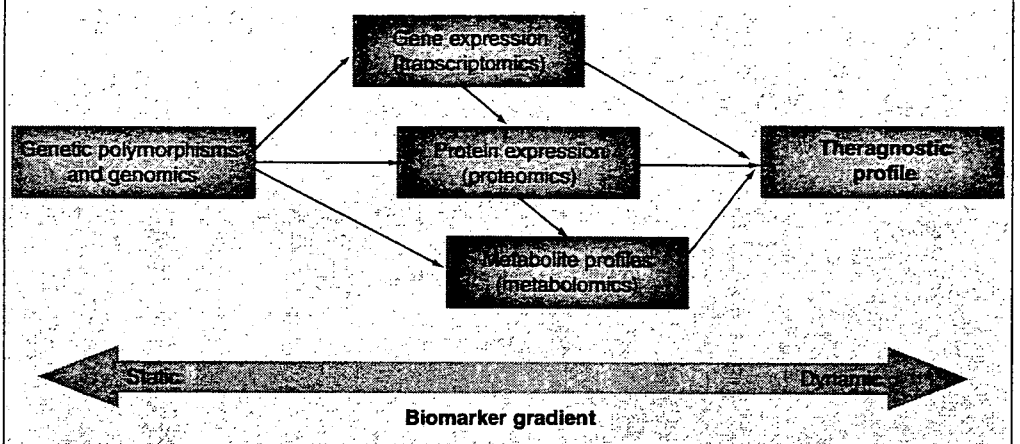
Figure 1. Hierarchy of biomarkers and their integration into theragnostic tests.



Hierarchy of biomarkers and their integration into theragnostic tests from gene sequence (upstream or static marker) to downstream (dynamic) markers on gene and protein expression or cellular metabolites. A theragnostic profile is depicted as a synthesis of various biomarker tests that characterize an individual patient and their drug treatment outcome. The theragnostic profile may be heuristic in nature when only a singular biomarker is associated with treatment outcomes while more mechanistic insights can be achieved when biomarkers from different levels of the biological hierarchy corroborate and complement each other (reproduced from [4] with permission).

overexpression; the test itself can use a variety of methods including gene (i.e., pharmacogenomic) and/or protein expression [7,8] Theragnostics is thus a more holistic approach (and not a singular technology) to diagnosis and therapy selection than has traditionally been the case in biomarker research or medical practice.

This paper identifies and differentiates two bottlenecks or gaps (hereafter referred to as GAP1 and GAP2) in the conduct of translational research in the emerging field of theragnostics. There is a major gap, GAP1, in the translation of basic science discoveries to first-in-human (FIH) proof-of-concept [9]. A second serious gap, GAP2, occurs in the transition from clinical proof-of-concept to the development of appropriate treatment guidelines and science policy. We suggest that resolution of these bottlenecks or gaps requires distinct research aims, resources and study designs. For example, research directed at GAP1 may require focused small sample size academic or industry-sponsored studies. In contrast, GAP2 translational research would require large-scale longitudinal population databases on observational 'real-life' treatment outcomes and core technical biomarker competency to explain variability in drug effects [10–12]. These gaps in translational research are collectively sufficiently important for the US FDA to have the view that, "the applied sciences needed for medical product development have not kept pace with the tremendous advances in the basic sciences. The new science is not being used to guide the technology development process in the same way that it is accelerating the technology discovery process" [11].

'Unpacking' translational research in theragnostics

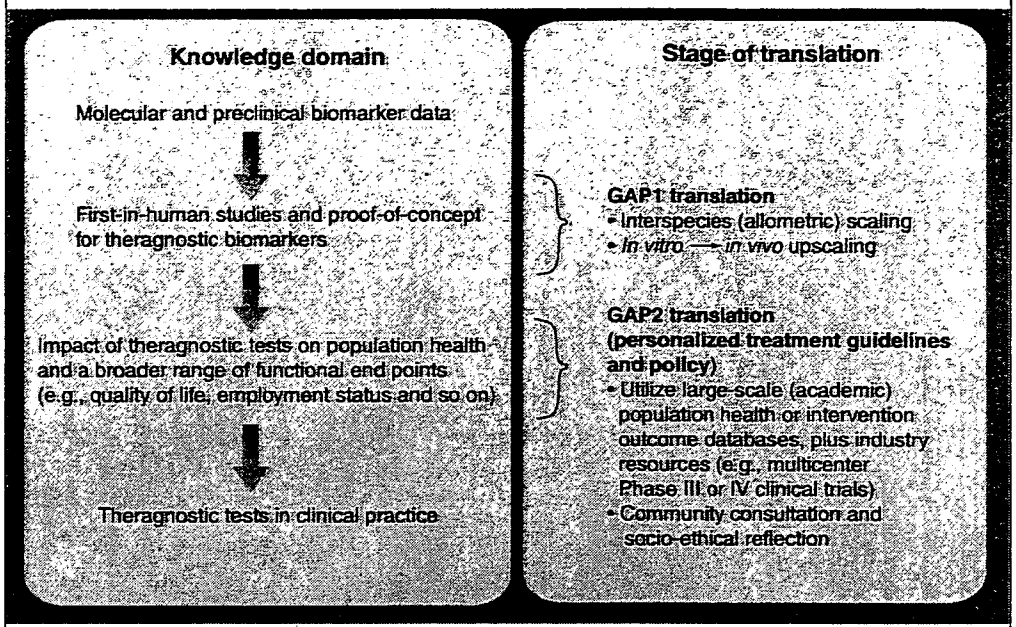
GAP1: translation from basic science to first-in-human proof-of-concept

The need for GAP1 translational research in theragnostics stems from three fundamental considerations:

- The obvious interspecies differences in pharmacokinetic pathways and molecular drug targets;
- The inevitable biological contrasts between the inbred laboratory animals with a homogenous genetic background and outbred human populations who exhibit marked genetic variability and exposure to a diverse array of social and environmental factors;
- The need for scaling up molecular observations *in vitro* to an integrated systems biology context in the whole (human) organism *in vivo* (Figure 2).

FIH proof-of-concept studies play a pivotal role in bridging the divide (i.e., GAP1) between preclinical biomarker research and large-scale population-based clinical investigations for theragnostic test development and validation. Despite their small sample size and limited scope

Figure 2. The projected two stages in translational biomarker research in theragnostic medicine.



The projected two stages in translational biomarker research in theragnostic medicine from clinical proof-of-concept (GAP1) to development of appropriate treatment guidelines based on theragnostic tests (GAP2).

of inquiry (usually less than 100 subjects per study), FIH studies make an important contribution as a first step in proof-of-concept and knowledge translation between *in vitro* and *in vivo* approaches, or more broadly, in extrapolation of data from animal models to the whole human organism. For example, clinical trials selectively testing patients with certain genetic subtypes of drug targets previously shown to confer an increased likelihood of response can facilitate proof-of-concept decisions on whether and to what extent a new molecular entity (NME) is a viable therapeutic candidate. An inadequate clinical response to an NME in such enriched samples may serve as an early indication of possible therapeutic failure in the general patient population [9].

A glance at leading clinical pharmacology and pharmacogenomic journals attests to the proliferation of genotype–phenotype correlative studies over the past 10 years [13,14]. Many of these studies fall under the GAP1 translational biomarker research; they often have small sample sizes. In an attempt to develop, implement, and disseminate a public genotype–phenotype resource, Stanford University (CA, USA), with funding from the NIH, established the Pharmacogenetics & Pharmacogenomics Knowledgebase (PharmGKB) [102]. This database is part of the NIH Pharmacogenetics Research Network (PGRN), a nationwide collaborative research consortium. The PharmGKB stores data regarding genetic sequence variation and their association with drug-related phenotypes, and provides methods for submission, browsing, and download. The PharmGKB is envisioned as an integrated research tool and repository for genetic, genomic, molecular and cellular phenotype data and clinical information on research participants in pharmacogenomics research studies. As of October 9, 2006, the PharmGKB reportedly contained information on 230 genes and its variants and 426 drugs. PharmGKB is comprised of clinical and basic pharmacokinetic and pharmacogenomic research data on, but not limited to, the cardiovascular, pulmonary and cancer pathways, and metabolic and transporter domains [102]. These data are publicly accessible on the internet for research purposes. In the short term, it is conceivable that biomarker data repositories such as PharmGKB will become an important aid to researchers in obtaining clinical proof-of-concept to understand how genetic variation among individuals contributes to differences in reactions to drugs. Looking further, such theragnostic databases may accumulate sufficient 'biomarker–phenotype' correlative studies to be able to inform population-based GAP2