important to understand how antidepressants change the brain in depressed patients.

Interestingly, antidepressants selectively affect certain immediate early genes and transcription factors, including c-fos (Dahmen et al., 1997; Torres et al., 1998), zif268 (Dahmen et al., 1997), NGFI-A (Bjartmar et al., 2000; Johansson et al., 1998), and Arc (activity-regulated role of cytoskeleton-associated protein) (Pei et al., 2000). In addition, the cyclic adenosine monophosphate (cAMP) second messenger system is another pathway that could be involved in antidepressant action. Chronic administration of antidepressants up-regulates the cAMP pathway at several levels, including at points that increase expression and phosphorylation of the cAMP response element binding protein (CREB) (Thome et al., 2000). These molecules may be important for adaptive neuronal changes that occur as a result of chronic antidepressant treatment. Region-specific effects of chronic antidepressant treatment on the DNA-binding activities of CRE-, SP1- and GRE-binding elements have been previously reported in rat hippocampus and frontal cortex (Frechilla et al., 1998). In addition, chronic treatment with citalopram (a selective serotonin reuptake inhibitor, SSRI) induces time-dependent changes in the expression and DNA-binding activity of transcription factor AP-2 in rat brain (Berggard et al., 2003; Damberg et al., 2000).

Together, these data suggest that changes in gene expression may have a role in the mechanism underlying antidepressant action. Therefore, the identification and quantitation of changes in gene expression associated with chronic antidepressant treatment can pave the way for the discovery of novel molecular markers that would be useful for the diagnosis and treatment of depression. For example, Chardenot et al. (2002) recently described significantly enhanced expression of a protein associated with the peripheral-type benzodiazepine receptor (PRAX-1) in rat hippocampus after chronic antidepressant treatment.

2.2. High-throughput methods for gene expression analysis

Recent developments in molecular neurobiology provide new conceptual and experimental tools (i.e., "the open target screen approaches") to investigate, and facilitate understanding of the mechanisms by which antidepressants produce long-lasting alterations in brain function. The emerging techniques and powerful tools derived from the relatively new subfields of genomics and proteomics hold great promise for the identification–independent of any preconceived hypotheses–of genes and gene products that are altered by chronic antidepressant treatment or other effective therapeutic manipulations, such as electroconvulsive therapy (ECT). Pharmacogenomic tools, such as differential display PCR, serial analysis of gene expression (SAGE), total gene expression analysis (TOGA), representational difference analysis (RDA), cDNA microarrays, and GeneChip® (ffymetrics, Santa Clara, CA), are now being used to study antidepressant-elicited changes in gene expression (Table 1). Because these methods are unbiased, the potential exists for identifying completely new classes of drug targets. Novel biological approaches tapping novel hypotheses beyond the "monoamine hypothesis" will definitely cause paradigm shifts to emerge in future antidepressant research.

2.2.1. cDNA microarray and GeneChip®

The recently introduced microarray technique (e.g., cDNA microarray and GeneChip®) permits us to efficiently perform large-scale, coordinated monitoring of gene expression during different functional states found in control and treated animals. The ability to monitor changes in gene expression at the genomic scale would be valuable for identifying factors that contribute to complex neuronal processes associated with antidepressant treatment. This technology also enhances efforts to characterize the structure and function of these genes.

Using GeneChip®, Rausch et al. (2002) studied whole brain kinase mRNA expression in rats treated with antidepressants. Interestingly, protein kinase C (PKC)-delta, PKC-gamma, stress-activated protein kinase, cAMP-dependent protein kinase beta isoform, Janus protein kinase, and phosphofructokinase M are all chronically down-regulated in rats treated with two SSRIs citalopram and fluoxetine. In addition, Landgrebe et al. (2002) reported antidepressant-induced changes in gene expression using cDNA microarrays containing 3624 expressed sequence tags (ESTs); these represented murine genes expressed in the brain. They found that two different antidepressants, paroxetine (a SSRI) and mitrazepine (a noradrenergic and specific serotonergic antidepressant), down-regulated four common genes (a ribosomal protein and three genes of unknown function). These drugs, however, induced very different gene expression profiles. Taken together, these findings suggest that antidepressants with different pharmacologies can have the same molecular targets, but may act on these targets via different primary pathways.

In contrast, tricyclic antidepressants (TCAs) appear to have multiple molecular targets in vitro and in vivo. For example, chronic treatment with the TCA desipramine increases the expression of six genes and decreases the expression of two genes in primary cultures of rat hippocampal cells (Chen et al., 2003). One of the up-regulated genes encodes the neuronal growth cone marker, growth-associated-protein 43 (GAP-43). Interestingly, in situ hybridization also revealed that desipramine increased GAP-43 gene expression in dentate gyrus but not other

Table 1
Open target screen approaches for gene expression analysis

Differential display PCR
Serial analysis of gene expression (SAGE)
Total gene expression analysis (TOGA)
Representational difference analysis (RDA)
cDNA microarrays/GeneChip®

brain regions (Chen et al., 2003). Because GAP-43 regulates growth of axons and modulates the formation of new connections, these findings suggest that desipramine may affect neuronal plasticity in the central nervous system.

Interestingly, noradrenaline also may have multiple molecular targets. Laifenfeld et al. (2002) previously found that noradrenaline treatment increased GAP-43 expression and progressively decreased Oct4 expression in human neuroblastoma SH-SY5Y cells. Using cDNA microarrays, this group also reported increased expression of two neurite-outgrowth promoting genes: neural cell adhesion molecule L1 and laminin. In addition, noradrenaline-treated SH-SY5Y cells display elongated, granule-rich somata and an increased number of neurites. Moreover, in the presence of noradrenaline, cell survival is enhanced, while proliferation is inhibited. Taken together, these results support a role for noradrenaline in processes associated with synaptic connectivity and in mediating the hypothesized neuronal plasticity associated with antidepressant treatment.

By combining genomic tools with sophisticated animal model systems, it will be possible to identify patterns of altered gene expression that are associated with particular features of antidepressant-induced and adaptative changes in the brain. Using SAGE and GeneChip® analysis, Feldker et al. (2003) reported differential expression of several genes in the hippocampus of genetically selected long attack latency (LAL) and short attack latency (SAL) mice. LAL and SAL mice differ in a wide variety of behavioral traits and display differences in the serotonergic system and the hypothalamus–pituitary–adrenocortical (HPA) axis. LAL mice exhibit elevated expression of numerous cytoskeleton genes, such as cofilin and several tubulin isotypes. LAL mice also show elevated expression of several calmodulin-related genes and genes encoding components of the MAPK cascade (e.g., raf-related oncogene and ERK2). These results suggest that differential regulation of the raf/ERK pathway may be related to structural differences in the hippocampus of LAL and SAL mice. As stress-related disorders, such as depression, are also linked to differential regulation of the HPA-axis and the serotonergic system and are associated with altered hippocampal, morphology, differential regulation of these genes may be involved in the pathogenesis of these diseases.

Recently, more and more large-scale expression analyses have been performed or are underway in several laboratories. In the next few years, pharmacogenomics will yield huge amounts of data about antidepressant-associated changes or depression-associated changes in gene expression that are relevant for the development of novel therapeutics. Therefore, it is very important to apply standardized criteria for experimental design and make primary data freely available for comparison and analysis.

2.2.2. *Differential display PCR*

Most known neuronal proteins (i.e., receptors, ion channels, and enzymes) were discovered previously either through biochemical isolation or traditional cloning methods, or through the examination of random sequences of cDNAs. Using a differential cloning strategy, we and other groups have isolated genes that are differentially expressed in the brain after chronic antidepressant treatment (Huang et al., 1997; Nishioka et al., 2003; Wong et al., 1996; Yamada et al., 1999, 2000, 2001, 2002). Independent of any preconceived hypothesis, these genes and proteins have been implicated in a physiological or pathophysiological process. For example, in the amygdala of rats that received daily treatment with the TCA imipramine for 3 weeks, the gene encoding a mutation suppressor for the Sec4–8 yeast (Mss4) transcript was overexpressed (Andriamampandry et al., 2002). This overexpression was also found in the hippocampus of rats treated chronically with two antidepressants having opposite molecular mechanisms of action, the serotonin reuptake enhancer tianeptine and the SSRI fluoxetine.

Using the RNA fingerprinting technique, a modified differential display PCR, we continue our efforts to identify biochemical changes induced by chronic antidepressant treatments. To date, we have cloned hundreds of cDNA candidates as ESTs from rat frontal cortex and hippocampus. Some of these candidate cDNAs should be affected by antidepressants and are thus named antidepressant-related genes (ADRGs).

The benefits and technical limitations of differential display PCR and cDNA microarrays are shown in Table 2. In addition to gene expression monitoring, the cDNA microarray method is useful for the identification of genes because it facilitates the screening of enriched libraries derived from experiments using differential cloning techniques. Therefore, to identify molecular machinery that mediate the therapeutic action of antidepressants, we developed an original cDNA microarray for ADRGs (ADRG microarray) (Yamada and Higuchi, 2002). To develop the ADRG microarray, each of the ADRGs were first amplified using PCR and then spotted in duplicate onto glass slides (Fig. 2) using the modified method of Salunga et al. (1999). Messenger RNA was purified from pooled total RNA using oligo-dT columns, and then converted to cDNA in the presence of either Cy-5 or Cy-3-dUTP to make fluorolabeled probes. Hybridization of the probes to the microarray was done competitively. The probes were mixed and placed on an array, overlaid with coverglass, and hybridized. After the hybridization and washing procedures, each slide was analyzed and gene expression levels were quantified using

Table 2
Comparison of two differential cloning techniques

(1) Differential display PCR
High rate of false positives, "needle-in-a-haystack" approach
Powerful tool to discover completely unknown genes/ESTs
Possible to detect novel splice variants
(2) cDNA microarrays
Ultra-high throughput analysis
Costly, limited accessibility for patented genes
Only genes/ESTs "spotted" on the microarray can be analyzed

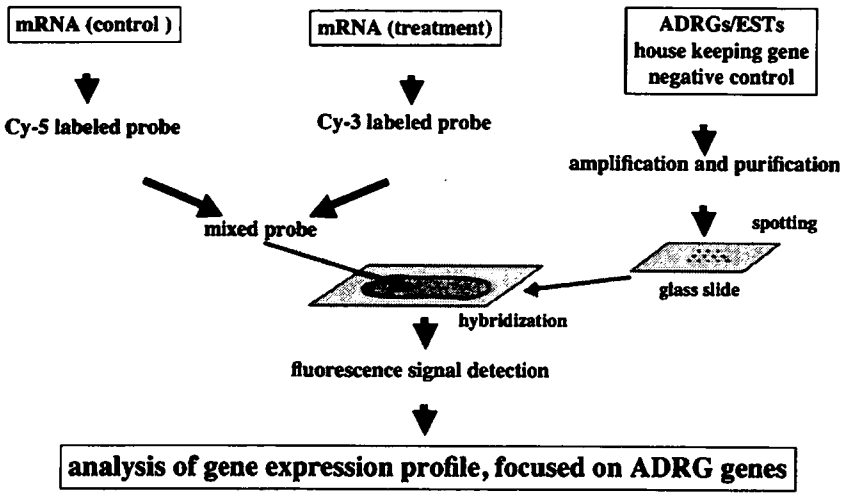

Fig. 2. Fabrication and analysis of ADRG microarray with fluorescent probes.

a PC and ImaGene software (Bio-Discovery Ltd., Swansea, UK). As expected, we obtained low background and consistent results in duplicate experiments. To date, we have identified several interesting genes and ESTs using the ADRG microarray and fluorolabeled probes. A pseudo-color image of an ADRG microarray is shown in Fig. 3.

After normalizing the fluorescent signals using both negative and positive controls, several spots of interest on our ADRG microarray showed increased or decreased fluorescence intensities following chronic antidepressant treatment (Nishioka et al., 2003; Yamada et al., 2000, 2001, 2002). The functional implications of these gene expression changes are currently under investigation.

2.3. Technical limitations and target validation

While rapid technical development of gene expression analysis raises high expectations for the future, many problems and limitations related to these methods need to be considered when interpreting data resulting from such analyses. Microarray analysis can typically identify relatively large differences (>2.0 fold). Because the brain is the most complex organ in our body with many different areas, nuclei, and cell types, the sensitivity of an expression array analysis may be insufficient to detect less abundant but physiologically important changes. Differential gene expression in the brain may also be masked if the mRNA is expressed by a large number of neurons but is regulated only in a subpopulation of these neurons. Furthermore, neuronal populations responsive to antidepressant treatment may be sparse or dispersed in brain. Therefore, the accuracy in dissection of specific brain regions for expression analysis is crucial to determine genuinely altered gene expression profiles. Attempts to reduce variation by examining a single well-defined brain nucleus, or even a single neuron, using laser capture microscopy have been introduced. These methods will have obvious advantages to reduce this limitation.

Gene expression analysis permits the identification of multiple regulated targets. Each target molecule must then be evaluated with traditional approaches (e.g., Northern analysis, quantitative RT-PCR, in situ hybridization, immunoblotting analysis, immunohistochemistry, etc.). Furthermore, data obtained by methods other than expression profiling

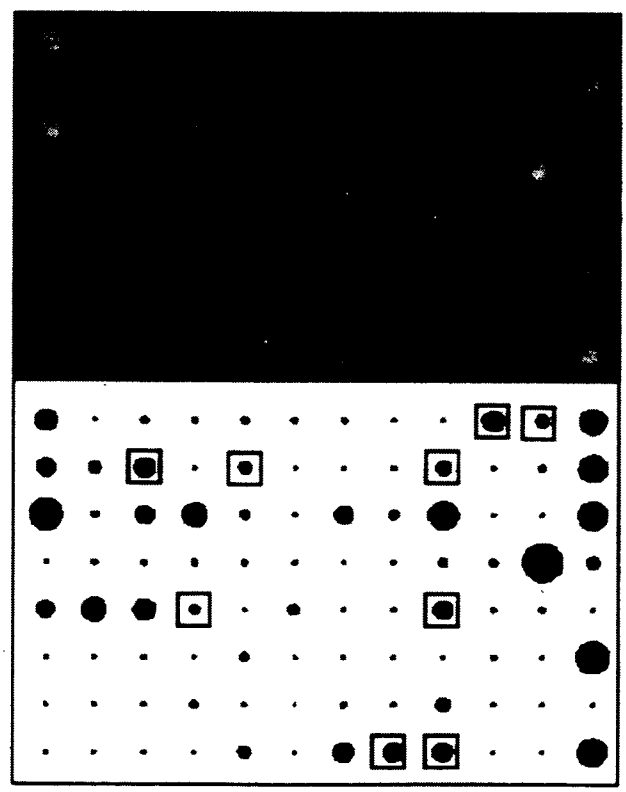

Fig. 3. Pseudo-color image of ADRG microarray after hybridization with fluorescent probes. Ninety-six spots representing ADRG 1–96 are shown here. The pseudo-color images of control group data (green) and antidepressant-treated group (red) were overlapped. The spots within the blue rectangles represent genes up-regulated by chronic antidepressant treatment. The spots within the pink rectangles represent genes down-regulated by chronic antidepressant treatment.

(such as genome sequence data, single nucleotide polymorphism data, homology data, molecular pathway data, etc.) must be combined with gene expression data to achieve a better understanding of protein function or drug treatments. In addition to pharmacological validations, novel drug targets can be validated by characterizing the neurobiological function of a target under normal conditions and by studying the target in behavioral models of depression. Unfortunately, animal models for depression are less straightforward when compared to those of other physical illnesses. Thus, despite all the best preclinical validation of a putative target of an antidepressant, it remains unclear whether "a drug aimed at the novel molecular target" will have the desired clinical effects in patients.

3. Neural plasticity, remodeling of neuronal circuits, and a new hypothesis

Many of the previous reports describing mechanisms of antidepressant action have focused on acute changes in synaptic pharmacology, especially on neurotransmitter turnover and neurotransmitter receptor changes. To advance our understanding of the therapeutic actions of antidepressants, we must now extend our efforts beyond theories based on the simple pharmacology of the synapse. This new effort must seek a deeper understanding of cellular and molecular neurobiology as well as examine the architecture and function of relevant neural systems. Many now believe that changes in brain gene expression, which are elicited after chronic antidepressant treatment, might underlie the drug-induced neural plasticity associated with the long-term actions of antidepressants in the brain and their clinical effects. Table 3 summarizes possible neuroplastic changes induced after chronic antidepressant treatment.

3.1. Survival of neurons and neurogenesis in the hippocampus

Although depression involves many psychological and social factors, it also represents a biological process: the effects of repeated exposure to stress on a vulnerable brain.

Table 3
Possible neuroplastic changes induced after chronic antidepressant treatment

(1) Functional neuroplastic changes
Vesicular docking/fusion/exocytotic machinery
Neurotransmitter release
Post-synaptic signal transduction system
(2) Morphological neuroplastic changes (remodeling of the neural circuits)
Vesicular docking/fusion/exocytotic machinery
Sprouting, neurite outgrowth
Neurotrophic factors
Neuronal death and survival
Axon guidance
Neurogenesis and new neural circuits

Preclinical and clinical research has focused on the interactions between stress and depression and their effects on the hippocampus (Duman et al., 1999; McEwen, 2000). The hippocampus is one of several brain regions that, when exposed to stressful stimuli, can contribute to the emotional, cognitive, and vegetative abnormalities found in depressed patients. This region of the brain is also involved in the feedback regulation of the hypothalamus–pituitary–adrenal axis, the dysfunction of which is associated with depression (Young et al., 1991). Studies suggest that stress-induced atrophy and loss of hippocampal neurons may contribute to the pathophysiology of depression. Interestingly, hippocampal volume is decreased in patients with stress-related psychiatric illnesses, including depression and post-traumatic stress disorder (Sapolsky and Duman, 2000; Sheline et al., 1996).

In vitro and in vivo data provide direct evidence that brain-derived neurotrophic factor (BDNF) is one of the key mediators of the therapeutic response to antidepressants (D'Sa and Duman, 2002). BDNF promotes the differentiation and survival of neurons during development and in the adult brain, as well as in cultured cells (Memberg and Hall, 1995; Palmer et al., 1997; Takahashi et al., 1999). Stress decreases the expression of BDNF, and reduced levels could contribute to the atrophy and compromised function of stress-vulnerable hippocampal neurons. In contrast, antidepressant treatment increases the expression of BDNF in the hippocampus, and could thereby reverse the stress-induced atrophy of neurons or protect these neurons from further damage (Duman, 1998; Duman et al., 1997). These findings have resulted in the development of a novel model of the mechanism of antidepressant action and have suggested new targets for the development of therapeutic agents.

While hippocampal volume can decrease in disease, the hippocampus is also one of only a few brain regions where the production of neurons normally occurs throughout the lifetime of several species of animals, including humans (Eriksson et al., 1998). Hippocampal neurogenesis is influenced by several environmental factors and stimuli (Gould and Tanapat, 1999; Nilsson et al., 1999; van Praag et al., 1999). For example, both acute and chronic stress cause decreases in cell proliferation (Fuchs and Flugge, 1998). On the other hand, administration of several different classes of antidepressant, as opposed to non-antidepressant, agents increases the number of BrdU-labeled cells, indicating that this is a common and selective action of antidepressants (Malberg et al., 2000). In addition, recent evidence indicates that electroconvulsive seizures (an animal model of ECT in humans) can also enhance neurogenesis in rat hippocampus (Hellsten et al., 2002; Madsen et al., 2000; Scott et al., 2000). These findings raise the possibility that increased cell proliferation and increased neuronal number may be a mechanism by which antidepressant treatment mitigates stress-induced atrophy and loss of hippocampal neurons, and thus may contribute to the therapeutic actions of antidepressant treatment. Furthermore, increased formation

of new neurons in the hippocampus related to antidepressant treatment may lead to altered expression of genes specifically expressed in immature neurons. Therefore, observed changes in gene expression may reflect alterations in cell composition of the tissue rather than changes in individual neurons.

3.2. Neurotransmitter release and vesicular exocytotic machinery

Considerable evidence indicates that VAMP-2 is a key component of the synaptic vesicle docking/fusion machinery that forms the SNARE (soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor) complex (Bennett et al., 1992; Oyler et al., 1989; Trimble et al., 1988; Weis and Scheller, 1998). As shown in Fig. 4, the SNARE complex consists of proteins on the target membrane, called t-SNARE, and proteins on the vesicular membrane, called v-SNARE. SNAP-25 and syntaxin are t-SNARE proteins, whereas VAMP-2 is a v-SNARE protein. Fusion of vesicles with the plasma membrane leads to exocytosis, which mediates the release of neurotransmitter into the synapse. Previously, we demonstrated a significant increase of both VAMP2 mRNA and protein levels in rat frontal cortex after chronic treatment with two different classes of antidepressants, imipramine and sertraline, and also with repeated ECT (Yamada et al., 2002). In this context, pharmacological modulation of VAMP2 gene expression would also be predicted to alter neurotransmitter release. Our data suggest that VAMP2 may be one of the common functional molecules induced after chronic antidepressant treatment. Interestingly, the work of others shows that acute and chronic administration of antidepressants diminishes the release of glutamate and aspartate, and inhibits veratridine-evoked 5-HT release (Golembiowska and Dziubina, 2000). An important feature of the action of antidepressants and ECT is that they do not globally alter the expression of other membrane-trafficking proteins. In contrast to the enhanced expression of VAMP2, we detected no significant change in the expression of other synaptic vesicle proteins (syntaxin-1 and SNAP-25). Although there are more than a dozen synaptic vesicle proteins (Sudhof, 1995), we chose to investigate the expression of syntaxin-1 and SNAP-25 because they make a SNARE-complex with VAMP2 and mediate the synaptic vesicle docking/fusion machinery. We reasoned that a coordinated change of VAMP2 and the expression of syntaxin-1 and SNAP-25 might signal a change in the overall number of SNARE complexes. An antidepressant-induced change in the expression of syntaxin-1 and SNAP-25, associated predominantly with the presynaptic plasma membrane, would have been indicative of more complex changes in the transmitter secretory pathway, such as an increase in the number of active zones. Instead, the absence of such a coordinated change in syntaxin-1 and SNAP-25 expression indicates that antidepressants or ECT produces a more selective modification of the regulated secretory machinery. Additional work will be necessary to understand the role of selective VAMP2 induction in rat frontal cortex.

As mentioned above, the CREB phosphorylation pathway could be involved in antidepressant action. Among the multiple target genes that could be regulated by CREB is BDNF (Duman, 1998; Duman et al., 1997). BDNF promotes long-term potentiation at hippocampal CA1 synapses via a presynaptic enhancement of synaptic transmission during high-frequency stimulation (HFS). Pozzo-Miller et al. (1999) showed that heterozygous mice with BDNF knockout display more pronounced synaptic fatigue

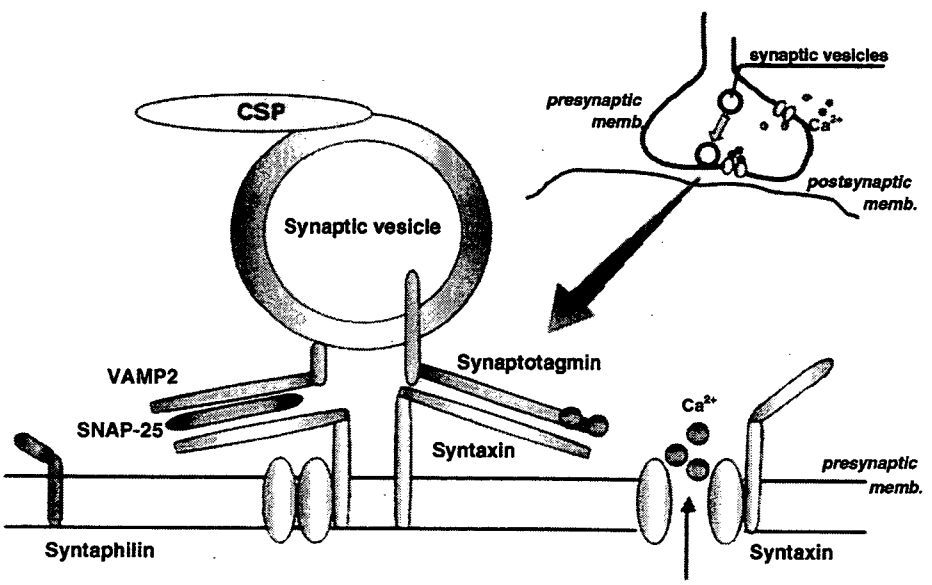

Fig. 4. SNARE complex at synapses.

at CA1 hippocampal synapses upon HFS, suggesting an impairment in transmitter release. This was associated with a decrease in the number of synaptic vesicles docked to the active zones (with no changes in the reserve pool of vesicles), and a decrease in the synaptic (not total) expression of VAMP2 and synaptophysin, respectively. Therefore, BDNF appears to be directly involved in the regulation of protein machinery at presynaptic terminals, an action consistent with its short- and long-term modulation of synaptic transmission. Recently, the same authors have found that long-term treatment of hippocampal slices with BDNF increases the number of docked vesicles at CA1 synapses, without altering the reserve pool, and greatly increases the expression of synaptotagmin, synaptophysin, and VAMP2 (Tartaglia et al., 2001).

Previously, we demonstrated that a unique cysteine-rich protein, called cysteine string protein (CSP), is clearly elevated in rat brain after chronic antidepressant treatment (Yamada et al., 2001). CSP was originally identified as a family of nervous system-specific antigens in *Drosophila*. CSP is localized to synaptic vesicle membranes (Gundersen and Umbach, 1992). Several reports indicate that CSP functions in the central nervous system to modulate the activity of presynaptic calcium channels, resulting in neurotransmitter release at the nerve terminal (Chamberlain and Burgoyne, 1998; Umbach et al., 1994). Consistent with these findings is a recent report showing that antibodies against CSP inhibit evoked neurotransmitter release at the *Xenopus* neuromuscular junction (Poage et al., 1999). In rat brain, CSP interacts with VAMP2 in synaptic vesicle membranes (Chamberlain and Burgoyne, 2000; Leveque et al., 1998). Taken together, this coordinated induction of two presynaptic molecules suggests that the number of secretory organelles, which includes both small clear vesicles as well as large dense-core granules, might be increased after chronic antidepressant treatment.

In addition, as described above, the expression of Mss4 is increased in the rat amygdala and hippocampus after chronic antidepressant treatment (Andriamampandry et al., 2002). Mss4 protein has the properties of a guanine nucleotide exchange factor, and interacts with several members of the Rab family implicated in Ca^{2+}-dependent exocytosis of neurotransmitters. Interestingly, Mss4 transcripts were specifically down-regulated in the hippocampus and amygdala of rats after exposure to chronic, mild stress. These findings suggest that gene expression-dependent alterations of neuronal transmitter release may be an important component of the pharmacological action of antidepressants.

It is reasonable to assume that alterations of mood, neurovegetative signs, or even social behavior of depressed patients reflect some changes in patterns of synaptic activity in the brain. Thus, it will be of interest to determine whether these changes in the brain contribute to clinical effects in patients treated with antidepressants. Additional work will be necessary to test this hypothesis.

3.3. Neurite outgrowth and sprouting

Interestingly, vesicular docking/fusion at the plasma membrane is responsible not only for the release of neurotransmitters, but also for surface expression of plasma membrane proteins and lipids. Therefore, exocytosis plays a fundamental role in axonal and dendritic outgrowth because both processes involve major increases in the surface area of the plasma membrane (Martinez-Arca et al., 2001). Several reports demonstrate that the SNARE complex has an important role in neurite outgrowth. For instance, inhibition of SNAP-25 expression by antisense oligonucleotides prevents neurite elongation in rat cortical neurons and neural crest-derived rat phenochromocytoma (PC12) cells in vitro (Morihara et al., 1999; Osen-Sand et al., 1996). Cleavage of SNAP-25 with botulinum neurotoxin A inhibits axonal growth (Morihara et al., 1999; Osen-Sand et al., 1996). Overexpression of SNAP-25 increases the number of neurites in nerve growth factor (NGF)-differentiated PC12 cells (Shirasu et al., 2000), and it has been reported that overexpression of syntaxin 1A inhibits NGF-induced neurite extension (Zhou et al., 2000). On the other hand, it has also been reported that overexpression of syntaxin 1A neither promotes nor inhibits neurite outgrowth in NGF-differentiated PC12 cells (Shirasu et al., 2000). This latter point still needs to be resolved. Inhibition of syntaxin 1A with antisense oligonucleotides or antibodies increases neurite sprouting and neurite length in rat dorsal root ganglion neurons, as well as in retinal ganglion neurons (Yamaguchi et al., 1996). It has also been reported that cleavage of syntaxin by botulinum neurotoxin C1 inhibits axonal growth (Igarashi et al., 1996). Several reports demonstrate that VAMP-2 also has an important role in neurite outgrowth; however, there are several discrepancies in these studies, and the detailed mechanism of VAMP-2 in neurite outgrowth is still unclear.

As mentioned above, chronic antidepressant treatment increases the expression of GAP-43 in the rat dentate gyrus (Chen et al., 2003). Laifenfeld et al. (2002) have also demonstrated that noradrenaline treatment results in an increase in GAP-43 in human neuroblastoma SH-SY5Y cells. Because GAP-43 regulates growth of axons and modulates the formation of new connections, these findings suggest that chronic antidepressant treatment may have an effect on structural neuronal plasticity in the central nervous system. Laifenfeld et al. (2002) have also reported an

Table 4
Novel candidate genes and molecular systems

(1) Neurogenesis
HPA axis and related neuroendocrine systems
Cyclic AMP second messenger system
Phosphorylation of CREB, BDNF
(2) Neurotransmitter release and neurite outgrowth
Cysteine string protein, VAMP2
Mss4, GAP-43, neural cell adhesion molecule L1, and laminin

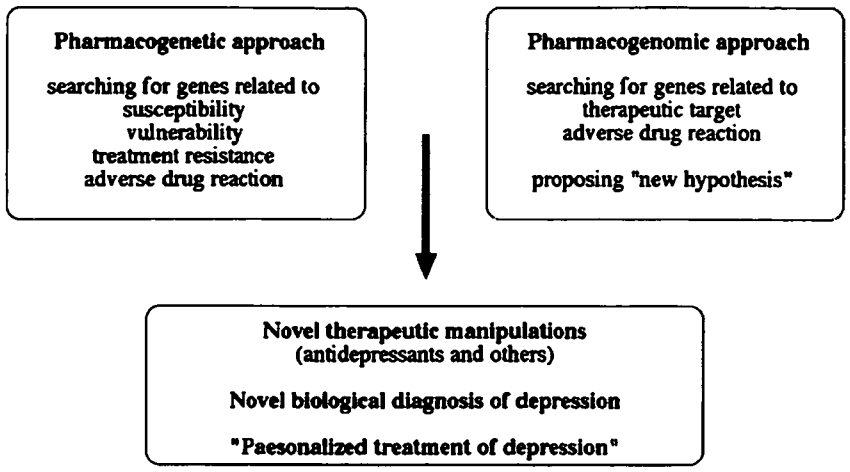

Fig. 5. Pharmacogenetics and pharmacogenomics in depression research.

increase in the expression of two neurite-outgrowth promoting genes: neural cell adhesion molecule L1 and laminin. Along with these effects, SH-SY5Y cells treated with noradrenaline had elongated granule-rich somas and increased numbers of neurites, when compared with non-treated cells. Moreover, cell survival was enhanced in the presence of noradrenaline, while proliferation was inhibited. Taken together, the results support a role for noradrenaline in processes of synaptic connectivity, and may point to a role of this neurotransmitter in mediating the hypothesized neuronal plasticity in antidepressant treatment. More recently, Dihne et al. (2003) reported that L1 influences proliferation and differentiation of neural precursor cells.

ECT is a safe and the most effective treatment for severely depressed patients who are resistant to antidepressant medications. Interestingly, the common effects of antidepressants and ECT on connectivity and synaptic plasticity in the dentate gyrus are likely to relate to affective functions of depression (Stewart and Reid, 2000). Consistent with these findings are data demonstrating that chronic electroconvulsive seizure administration in animals induces sprouting of the granule cell mossy fiber pathway in the hippocampus (Vaidya et al., 1999).

4. Conclusion

In the present review, we demonstrated that certain novel candidate genes may underlie the mechanism of action of antidepressants (Table 4). The limiting factor for the development of new treatments for depression is the paucity of novel targets. Identification of such targets will advance future efforts in the quest to develop effective therapeutics that have a new mode of action in the brain. In addition, we still do not know why only some depressed patients respond to treatments and others do not. Our future challenge is to identify the variations in specific human candidate genes that make some depressed individuals more vulnerable to social and biological stressors, or resistant to antidepressant treatment. The pharmacogenomics approach for predicting drug responsiveness will soon be adopted as the standard practice for the development of many drugs. Such detailed knowledge will offer novel insights into the actions of antidepressants that may be of both basic and clinical significance (Fig. 5).

In conclusion, functional genomics beyond the "monoamine hypothesis" will most likely cause paradigm shifts in antidepressant research in the future. Here, we propose the hypothesis that "remodeling of neuronal circuits" could be the basis for the action of antidepressants.

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Polymorphisms in the 5-Hydroxytryptamine 2A Receptor and Cytochrome*P*4502D6 Genes Synergistically Predict Fluvoxamine-Induced Side Effects in Japanese Depressed Patients

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5-Hydroxytryptamine (5-HT) receptors are thought to be associated with the gastrointestinal side effects induced by selective serotonin reuptake inhibitors. Cytochrome*P*450 (CYP) 2D6 may also be associated with the side effects induced by fluvoxamine, since the plasma fluvoxamine concentration depends on a CYP2D6 gene polymorphism. This study investigated whether 5-HT receptor and CYP2D6 gene polymorphisms could predict the occurrence of the side effects. The effects of 5-HT receptor and CYP2D6 gene polymorphisms on the incidence of gastrointestinal side effects induced by fluvoxamine were investigated in 100 depressed outpatients who gave written consent to participate in the study. The patients visited every 2 weeks until the week 12 end point and the fluvoxamine dose was changed in response to their clinical symptoms. All side effects, including the gastrointestinal side effects, were assessed at each visit. Polymerase chain reaction was used to determine A-1438G of the 5-HT2A receptor, C195T and Pro16Ser of the 5-HT3A receptor, Tyr129Ser of the 5-HT3B receptor, and the *5 and *10 alleles of CYP2D6. Both the A-1438G polymorphism of the 5-HT2A receptor gene and the CYP2D6 gene polymorphism had significant effects on the incidence of gastrointestinal side effects. Cox regression was used to analyze the combination effect of the two polymorphisms on the gastrointestinal side effects. Cox regression analysis showed that lower metabolizers (LMs) of CYP2D6 with the G/G genotype of the 5-HT2A A-1438G polymorphism had a 4.242-fold ($P=0.009$) and LMs with the A/G genotype had a 4.147-fold ($P=0.004$) higher risk of developing gastrointestinal side effects than normal metabolizers with the A/A genotype. The 5-HT3A and 3B gene polymorphisms had no significant effects on the incidence of gastrointestinal side effects. 5-HT2A receptor and CYP2D6 gene polymorphisms had a synergistic effect for the prediction of fluvoxamine-induced gastrointestinal side effects.

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INTRODUCTION

Fluvoxamine is widely available in Europe and the United States, and was introduced into clinical use as the first approved selective serotonin (5-hydroxytryptamine (5-HT)) reuptake inhibitor (SSRI) in Japan in 1999. Although SSRIs, including fluvoxamine, are known to have fewer side effects than tricyclic antidepressants (TCAs), the side-effect profiles of SSRIs and TCAs are different (Trindade *et al*, 1998). While TCAs have been reported to cause anticholinergic side effects, including a dry mouth, constipation, blurred vision, urinary retention, and postural hypotension, SSRIs are associated with gastrointestinal side effects, including nausea, vomiting, stomach ache, and diarrhea. In general, SSRIs are better tolerated than TCAs, although the gastrointestinal side effects have an incidence of up to 40% (Kasper *et al*, 1992; Trindade *et al*, 1998) and can be severe enough to lead to early treatment discontinuation (Kasper *et al*, 1992; Trindade *et al*, 1998; Murphy *et al*, 2003).

Recently, 5-HT3 receptors have been considered to have important roles in SSRI-induced gastrointestinal side effects, since the 5-HT3 receptor antagonists cisapride and ondansetron were reported to reduce the gastrointestinal side effects induced by SSRIs (Bergeron and Blier, 1994). Some variations in the 5-HT3A and 5-HT3B receptor genes have been detected (Tremblay *et al*, 2003; Kaiser *et al*, 2004), and Tremblay *et al* (2003) reported that variations in the

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5-HT3B receptor gene predicted the efficacy of antiemetic treatment in cancer patients. However, no previous studies have investigated the effects of 5-HT3A and 5-HT3B receptor gene polymorphisms on the occurrence of gastrointestinal side effects induced by SSRIs.

Furthermore, since peripheral 5-HT2A receptors are associated with gut motility and vascular smooth muscle tone (Banes *et al*, 1999; Janssen *et al*, 2002), polymorphisms of the 5-HT2A receptor gene may affect the gastrointestinal side effects induced by SSRIs. Murphy *et al* (2003) reported that the T102C polymorphism of the 5-HT2A receptor gene could predict the treatment discontinuation caused by paroxetine-induced side effects in depressed elderly patients. However, since elderly patients are considered to have different pharmacodynamic and pharmacokinetic profiles from younger patients, it is necessary to investigate whether the results in Murphy *et al* (2003) are consistent with those for other depressed patients.

Kasper *et al* (1992) reported that an increased incidence of nausea was associated with higher plasma concentrations of fluvoxamine. Cytochrome*P*450 (CYP) 2D6 has been shown to be involved in the metabolism of fluvoxamine, and CYP2D6 is known to have genetic polymorphisms that affect the enzyme activity (Greenblatt *et al*, 1998; http://www.imm.ki.se/CYPalleles/). These observations suggest that the polymorphic CYP2D6 may be a predictor for fluvoxamine-induced side effects. On the other hand, Hartter *et al* (1998) reported that there was no relationship between the serum concentration of fluvoxamine and the side effects. Gerstenberg *et al* (2003) reported that steady-state plasma concentrations of fluvoxamine were not associated with the incidence of nausea, and that the CYP2D6 genotypes did not affect nausea development. Further studies are needed to clarify whether the CYP2D6 gene polymorphisms affect fluvoxamine-induced side effects.

In this study, we investigated the effects of pharmacodynamic factors, such as 5-HT2A, 5-HT3A, and 5-HT3B receptor gene polymorphisms, and pharmacokinetic factors, such as CYP2D6 genotypes, on the occurrence of gastrointestinal side effects induced by fluvoxamine in Japanese depressed patients.

MATERIALS AND METHODS

Subjects

This study was conducted at the Niigata University Medical Hospital. The study protocol was approved by the Ethics Committee of Niigata University Medical Hospital, and each subject provided written informed consent before enrolment. The subjects comprised 100 Japanese depressed outpatients (47 men, 53 women) aged 40.5±15.7 years (mean age±SD). In all, 85 subjects had DSM-IV diagnoses of major depressive disorder, seven had adjustment disorder with depressed mood, six had a depressive disorder not otherwise specified, and two had bipolar I disorder in a depressed state. The exclusion criteria were additional diagnoses of Axis I or II of DSM-IV. All the patients had been free from psychotropic drugs for at least 14 days before their entry into the study. Demographic data, medical histories, and laboratory data, including hematology, serology, electrolytes, and urine analysis, were collected for each patient. Patients with obvious physical illnesses were excluded from the study. All patients were orally treated with fluvoxamine for their psychiatric illnesses.

Study Design

On the first examination (week 0), after informed consent was obtained, the symptoms of the patients were evaluated by the 17-item Hamilton Rating Scale for Depression (HAM-D-17) and they were treated with fluvoxamine at a starting dose of 25 mg/day for the first week. The patients subsequently visited at weeks 1, 2, 4, 6, 8, 10, and 12 after the first examination. The HAM-D-17 score and all side effects, including the gastrointestinal side effects, were assessed at each visit. If the improvement rate in the HAM-D-17 score was less than 40% compared with the score on the previous visit, the fluvoxamine dose was increased from 25 to 50 mg/day, and subsequently to 100, 150, and 200 mg/day if necessary. When the patients achieved remission (a HAM-D-17 score of less than 8 points), the fluvoxamine dose was not subsequently changed. Side effects were evaluated by our original rating scale, including 13 items as follows: nausea, vomiting, dry mouth, anorexia, constipation, diarrhea, stomachache, sleepiness, irritable mood, anxiety, insomnia, headache, and dizziness. Subjects were interviewed about the 13 items at each visit and the severity of each item was evaluated according to the two-grade (0 or 1) system. Gastrointestinal side effects consisted of five items: nausea, vomiting, anorexia, diarrhea, and stomachache. As gastrointestinal symptoms are not only side effects, but also symptoms of major depression, the gastrointestinal symptoms were not evaluated as side effects when the item 'gastrointestinal symptoms' of HAM-D-17 score become worse compared with the last visit.

Data Collection

Blood sampling was performed using a Venoject® tube containing EDTA-Na (Terumo Japan, Tokyo, Japan) at week 1 for genotype detection, and subsequently at the first appearance of gastrointestinal side effects to measure the concentration of fluvoxamine. Blood samples were also taken at 12 h after the final ingestion of fluvoxamine. In all, 7 ml of venous blood was collected, and genomic DNA was extracted from the peripheral leukocytes by utilizing a QIAamp Blood Kit (QIAGEN Inc., CA, USA) within 2 h of collection. Polymerase chain reaction (PCR) was used to determine the A-1438G genotype of the 5-HT2A receptor gene according to Erdmann *et al* (1996), the C195T and Pro16Ser genotypes of the 5-HT3A receptor gene according to Niesler *et al* (2001), and the Tyr129Ser genotype of the 5-HT3B receptor gene according to Tremblay *et al* (2003).

CYP2D6*10 alleles causing decreased enzyme activity were identified by the C188T mutation using a two-step PCR analysis as described (Johansson *et al*, 1994). A long-PCR analysis was used to detect the *5 allele causing a lack of enzyme activity as described (Steen *et al*, 1995).

The plasma concentration of fluvoxamine was measured using a column-switching high-performance liquid chromatography method with ultraviolet detection. The drug in

plasma, to which cisapride had been added as an internal standard, was extracted with hexane-chloroform, and the extract was subjected to automated column-switching high-performance liquid chromatography using a TSK BSA-C8 precolumn (Tosoh, Tokyo, Japan) for sample clean-up, and a TSK gel ODS-80TS column (Tosoh) for separation.

Statistical Analysis

Kaplan–Meier survival analysis and Cox regression analysis were used to compare the probabilities of the incidence of side effects. Genotype and allele distributions were analyzed by the χ^2-test. The clinical and demographic characteristics, onset weeks, onset doses, onset concentrations, and cumulative numbers of side effects were compared among groups by the unpaired *t*-test or one-way analysis of variance. The level of significance was set at less than 0.05.

RESULTS

The genotype frequencies of the 5-HT2A, 5-HT3A, and 5-HT3B genes are shown in Tables 1, 2 and 3. All of these genetic variations were in Hardy–Weinberg equilibrium. The genotypes of the 5-HT3A C195T polymorphism were not detected in seven patients. The other three genotypes of four patients could not be identified. No significant differences were demonstrated for sex, age, and baseline HAM-D-17 scores among the genotype groups.

Effect of the 5-HT2A Gene A-1438G Polymorphism

The cumulative incidences of fluvoxamine-induced gastrointestinal side effects are presented in Figure 1. Cox regression was used to analyze the effect of the 5-HT2A gene A-1438G polymorphism on the gastrointestinal side effects. The number of G alleles was entered into the analysis as an independent variable, and sex, age, and baseline HAM-D-17 score were added as potential confounders. The Cox regression analysis showed that patients with one G allele had a 2.171-fold higher risk of developing gastrointestinal side effects ($P=0.041$; 95% confidence interval (CI), 1.032–4.566) and patients with two G alleles had a 2.926-fold higher risk of developing gastrointestinal side effects ($P=0.008$; 95% CI, 1.321–6.481) than patients with no G allele. Sex, age, and baseline HAM-D-17 scores showed no significant effects on the risk of developing gastrointestinal side effects. There were no significant differences in the incidence of discontinuation between the three genotype groups ($\chi^2=0.029$, df=2, $P=0.986$) (Table 1).

Significant trends were demonstrated for the cumulative number of gastrointestinal side effects between the three genotype groups, although no significant differences were observed for the onset weeks, onset doses, and onset concentrations of fluvoxamine.

On the other hand, survival analyses showed no significant effect of the A-1438G polymorphism on the onset rate of all side effects, including the gastrointestinal side effects. No significant differences were demonstrated for the onset weeks, onset doses, onset concentrations, and cumulative numbers of all side effects between the genotype groups (Table 1).

Table 1 Characteristics of the Demographic Data and Fluvoxamine-Induced Side Effects by Comparison of the A-1438G Genotypes of the 5-HT2A Receptor

	5-HT2A gene A-1438G polymorphism[a]		
	A/A (N=28)	**A/G (N=41)**	**G/G (N=27)**
Sex (M/F)	12/16	24/17	11/16
Age	40.8 (17.6)	41.3 (15.4)	38.2 (14.7)
Baseline HAM-D-17 score	21.6 (5.2)	20.2 (5.4)	20.0 (4.9)
Discontinuation	7	11	7
P		0.986	
Gastrointestinal side effects			
Number of patients	10	25	18
Onset week	3.4 (2.9)	3.9 (2.7)	2.1 (2.0)
P		0.073	
Onset dose (mg)	60.0 (42.8)	75.0 (53.5)	45.8 (43.9)
P		0.16	
Onset concentration (ng/ml)	46.5 (70.3)	25.0 (27.8)	24.6 (32.9)
P		0.366	
Cumulative number of side effects	0.7 (1.2)	1.7 (2.0)	2.0 (2.7)
P		0.05	
All side effects			
Number of patients	21	34	22
Onset week	3.2 (2.9)	3.1 (2.8)	1.9 (1.7)
P		0.139	
Onset dose (mg)	56.0 (31.5)	62.5 (47.8)	39.8 (24.0)
P		0.097	
Cumulative number of side effects	2.8 (3.3)	5.3 (6.7)	5.0 (4.6)
P		0.147	

[a]Analysis stratified by the three genotype groups.
5-HT = 5-hydroxytryptamine; HAM-D-17 = 17-item Hamilton Rating Scale for Depression.

Effects of the 5-HT3A and 5-HT3B Gene Polymorphisms

Although Cox regression analysis was performed to investigate the effects of Pro16Ser and C195T of the 5-HT3A gene and Tyr129Ser of the 5-HT3B gene on the gastrointestinal side effects or all side effects, these polymorphisms had no significant effects on the occurrence of fluvoxamine-induced side effects (Tables 2 and 3). No significant differences were demonstrated for the onset weeks and doses of gastrointestinal side effects or all side effects in each genotype group.

Effect of the CYP2D6 Gene Polymorphism

The allele frequencies of the *5 and *10 alleles were 3.6 and 38.1%, respectively. The patients were divided into two genotype groups by the degree of enzyme activity: 75

Table 2 Characteristics of the Demographic Data and Fluvoxamine-Induced Side Effects by Comparison of the 5-HT3A Receptor Gene Polymorphisms

	5-HT3A gene					
	Pro16Ser			C195T		
	Pro/Pro (N=76)	Pro/Ser (N=18)	Ser/Ser (N=2)	C/C (N=8)	C/T (N=35)	T/T (N=50)
Sex (M/F)	38/38	9/9	0/2	2/6	18/17	25/25
Age	41.2 (15.5)	39.1 (16.2)	17.0 (5.7)	29.3 (15.5)	38.5 (13.3)	43.5 (16.6)
Baseline HAM-D-17 score	20.1 (5.2)	21.8 (5.2)	24.5 (0.7)	22.3 (5.7)	19.9 (5.1)	20.6 (5.3)
Gastrointestinal side effects						
Number of patients	44	7	2	5	19	26
All side effects						
Number of patients	63	12	2	6	29	39

5-HT = 5-hydroxytryptamine; HAM-D-17 = 17-item Hamilton Rating Scale for Depression.

Table 3 Characteristics of the Demographic Data and Fluvoxamine-Induced Side Effects by Comparison of the 5-HT3B Receptor Gene Polymorphisms

	5-HT3B gene		
	Tyr129Ser		
	Tyr/Tyr (N=54)	Tyr/Ser (N=37)	Ser/Ser (N=5)
Sex (M/F)	29/25	17/20	1/4
Age	40.2 (16.6)	39.8 (15.4)	44.4 (9.9)
Baseline HAM-D-17 score	21.2 (5.2)	20.1 (5.3)	17.2 (4.3)
Gastrointestinal side effects			
Number of patients	28	22	3
All side effects			
Number of patients	42	30	5

5-HT = 5-hydroxytryptamine; HAM-D-17 = 17-item Hamilton Rating Scale for Depression.

patients with the *1/*1 or *1/*10 genotype were termed normal metabolizers (NMs), and 22 patients with the *10/*10, *1/*5 or *5/*10 genotype were termed lower metabolizers (LMs) (Table 4). Figure 2 shows the effect of the CYP2D6 polymorphism on the incidence of gastrointestinal side effects. Cox regression analysis showed that LMs of CYP2D6 had a significantly higher risk of developing gastrointestinal side effects than NMs ($P=0.043$; hazard ratio (HR), 1.821; 95% CI, 1.019–3.254).

There were no significant differences in the incidence of discontinuation between NMs and LMs ($\chi^2=1.029$, df = 1, $P=0.310$). No significant differences were demonstrated for the onset weeks, onset doses, onset concentrations, and cumulative numbers of gastrointestinal side effects or all side effects between NMs and LMs (Table 4).

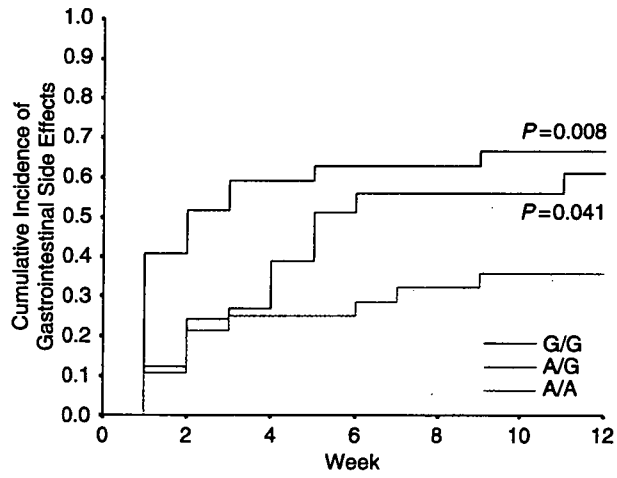

Figure 1 Effect of the A-1438G polymorphism of the 5-HT2A receptor gene on the cumulative 12-week incidence of gastrointestinal side effects induced by fluvoxamine.

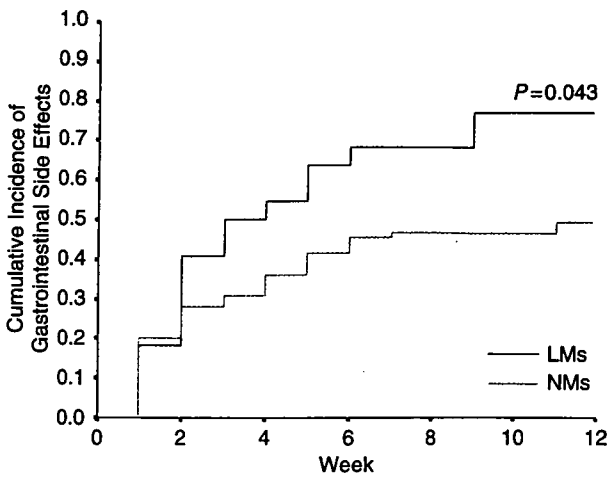

Figure 2 Effect of the CYP2D6 genotype on the cumulative 12-week incidence of gastrointestinal side effects induced by fluvoxamine.

Table 4 Characteristics of the Demographic Data and Fluvoxamine-Induced Side Effects by Comparison of the CYP2D6 Genotype Groups

	CYP2D6 genotype (phenotype)[a]		
	*1/*1, *1/*10 (NMs) (N = 75)		*1/*5, *10/*10, *5/*10 (LMs) (N = 22)
Sex (M/F)	35/40		12/10
Age	39.1 (16.1)		43.8 (14.2)
Baseline HAM-D-17 score	20.3 (5.3)		21.4 (5.0)
Discontinuation	17		8
P		0.267	
Gastrointestinal side effects			
Number of patients	37		17
Onset week	3.1 (2.7)		3.4 (2.6)
P		0.723	
Onset dose (mg)	64.9 (50.5)		58.8 (47.6)
P		0.679	
Onset concentration (ng/ml)	29.7 (44.8)		27.4 (30.3)
P		0.855	
Cumulative number of side effects	2.7 (2.3)		2.8 (1.9)
P		0.849	
All side effects			
Number of patients	58		20
Onset week	2.7 (2.4)		3.3 (3.2)
P		0.357	
Onset dose (mg)	56.0 (37.3)		51.3 (44.0)
P		0.638	
Cumulative number of side effects	5.3 (4.5)		6.8 (7.7)
P		0.285	

[a]Analysis stratified by the two genotype groups.
CYP = CytochromeP450; NMs = normal metabolizers; LMs = lower metabolizers; HAM-D-17 = 17-item Hamilton Rating Scale for Depression.

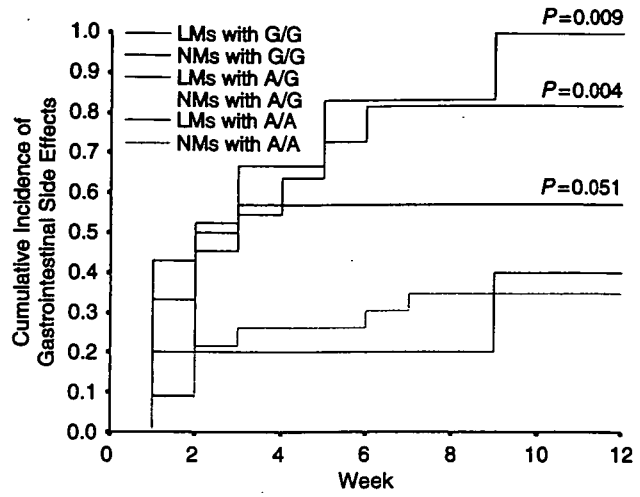

Figure 3 Combination effect of the A-1438G polymorphism of the 5-HT2A receptor gene and the CYP2D6 gene polymorphism on the cumulative 12-week incidence of gastrointestinal side effects induced by fluvoxamine.

Table 5 Combination Effect of the A-1438G Polymorphism of the 5-HT2A Receptor and the CYP2D6 Phenotype

	Hazard ratio	95% CI	P
Sex	0.65	0.368–1.146	0.136
Age	0.986	0.968–1.005	0.148
Baseline HAM-D-17 score	1.045	0.988–1.106	0.124
Combination of 5-HT2A and CYP2D6 polymorphisms			
LMs with A/A	0.859	0.179–4.122	0.849
NMs with A/G	1.681	0.717–3.939	0.232
LMs with A/G	4.147	1.558–11.038	0.004*
NMs with G/G	2.491	0.997–6.223	0.051
LMs with G/G	4.242	1.444–12.459	0.009*

*Statistically significant.
5-HT = 5-hydroxytryptamine; CYP = CytochiomeP450; HAM-D-17 = 17-item Hamilton Rating Scale for Depression; NMs = normal metabolizers; LMs = lower metabolizers.

Combination Effects of 5-HT2A Receptor and CYP2D6 Gene Polymorphisms

The above results indicated that both the A-1438G polymorphism of the 5-HT2A receptor gene and the CYP2D6 gene polymorphism had significant effects on the incidence of gastrointestinal side effects. Therefore, Cox regression was used to analyze the combination effect of the two polymorphisms on the gastrointestinal side effects. Figure 3 shows the combination effect of the 5-HT2A receptor and CYP2D6 gene polymorphisms on the incidence of the gastrointestinal side effects. The Cox regression analysis showed that LMs of CYP2D6 with the G/G genotype had a 4.242-fold higher risk of developing gastrointestinal side effects ($P = 0.009$) and LMs with the A/G genotype had a 4.147-fold higher risk of developing gastrointestinal side effects ($P = 0.004$) than NMs with the A/A genotype (Table 5). NMs with the G/G genotype had a 2.491-fold higher risk of developing gastrointestinal side effects ($P = 0.051$) than NMs with the A/A genotype (Table 5). Sex, age, and baseline HAM-D-17 scores showed no significant effects on the risk of developing gastrointestinal side effects.

DISCUSSION

In this study of a Japanese sample population, it was first demonstrated that the A-1438G polymorphism of the 5-HT2A receptor gene might predict the incidence of gastrointestinal side effects induced by fluvoxamine in depressed patients. Murphy *et al* (2003) reported that discontinuation due to paroxetine-induced side effects was

strongly associated with the C/C genotype of the 5-HT2A gene T102C polymorphism, and that there was a significant linear relationship between the number of C alleles and the probability of discontinuation. Since T102C is in complete linkage disequilibrium with the A-1438G polymorphism, the results in Murphy *et al* (2003) are generally consistent with those reported in this study. However, there are some important differences between the results in the two studies. Although the probability of discontinuation due to any adverse events, including gastrointestinal side effects, differed significantly between the genotype groups in the former study, our results showed significant differences between the genotype groups for the incidence of gastrointestinal side effects, but not for the incidence of all side effects. Furthermore, the A-1438G polymorphism had no significant effect on discontinuation in this study. Our results may indicate that the A-1438G polymorphism is only strongly related to the gastrointestinal side effects induced by fluvoxamine. While the subjects in the former study were 65 years of age or older, the mean age of the subjects in this study was 40.2 ± 15.7 years. Since elderly patients are thought to have pharmacodynamic and pharmacokinetic profiles different from those of younger patients, the difference in age may explain the discrepancy between the two studies. In addition, the difference in medication, paroxetine *vs* fluvoxamine, may cause disagreement of the results, since the two SSRIs have been reported to have different pharmacodynamic and pharmacokinetic profiles (Bourin *et al*, 2001). On the contrary, Yoshida *et al* (2003) reported that the A-1438G polymorphism of the 5-HT2A gene had no significant effect on the incidence of nausea. Their results disagree with those presented in this study. This may result from differences between the two studies in the numbers of subjects and other methodological points such as the dosage schedules and periods of observation.

A postmortem brain study found that the C allele of T102C (in complete linkage disequilibrium with the G allele of A-1438G) was associated with lower messenger ribonucleic acid (mRNA) and lower protein expression than the T allele (Polesskaya and Sokolov, 2002). Parsons *et al* (2004) reported that the presence of the A allele of A-1438G significantly increased promoter activity compared to the G allele. However, a study by Bray *et al* (2004) failed to replicate the differences in mRNA expression. Since the possible role of A-1438G in promoter function remains unclear, further studies are needed to clarify why this polymorphism affects the incidence of gastrointestinal side effects induced by fluvoxamine.

Moreover, sleep disturbances (Landolt *et al*, 1999) and sexual dysfunction (Sargent *et al*, 1998) are preferentially associated with the 5-HT2A receptor and it has been reported that SSRI-induced gastrointestinal side effects are mediated by the 5-HT3 receptor (Bergeron and Blier, 1994). To our knowledge, there have been no previous studies investigating the relationship between polymorphisms of the 5-HT3A and 3B genes and the gastrointestinal side effects induced by SSRI. In the current study, the polymorphisms of the 5-HT3A and 3B genes had no significant effects on the onset of fluvoxamine-induced gastrointestinal side effects. Tremblay *et al* (2003) reported that an insertion/deletion polymorphism in the promoter region of the 5-HT3B gene had a significant effect on the incidence of nausea and vomiting induced by cancer chemotherapy and that the Tyr129Ser polymorphism of the 5-HT3B gene, detected in this study, did not affect these side effects. On the other hand, Kaiser *et al* (2004) reported that polymorphisms of the 5-HT3A receptor gene may not serve as pharmacogenetic predictors of antiemetic treatment with 5-HT3 receptor antagonists in cancer patients. Since it is possible that polymorphisms of the 5-HT3A and 3B genes other than those detected in this study have significant effects on the gastrointestinal side effects, further studies are needed to clarify the impact of polymorphisms of the 5-HT3 gene on SSRI-induced gastrointestinal side effects.

Similar to A-1438G of the 5-HT2A receptor gene, the CYP2D6 polymorphism also showed a significant effect on the incidence of gastrointestinal side effects. Cox regression analysis showed that the combination of the A-1438G genotype and the CYP2D6 genotype could strongly predict the incidence of fluvoxamine-induced gastrointestinal side effects (Table 5). Indeed, there were six LMs of CYP2D6 who had the G/G genotype of the 5-HT2A receptor gene, and all of them suffered from gastrointestinal side effects. Among 11 LMs of CYP2D6 who had the A/G genotype, nine (81.8%) suffered from gastrointestinal side effects. In clinical situations, taking account of these results, tailor-made pharmacotherapy for fluvoxamine based on genetic factors may be possible. For example, LMs with the G/G or A/G genotype should be treated by antidepressants other than SSRIs or should be treated at lower starting doses of fluvoxamine than the other patients. Kasper *et al* (1992) reported that an increased incidence of nausea is associated with higher plasma concentrations of fluvoxamine. Since it has been shown that the plasma concentrations of fluvoxamine depend on the CYP2D6 polymorphism, our results support the preceding study. However, Murphy *et al* (2003) reported that the CYP2D6 genotype did not influence the side effects from paroxetine. Gerstenberg *et al* (2003) also reported that the number of mutated CYP2D6 alleles was not related to the development of nausea induced by fluvoxamine. CYP2D6 gene polymorphisms are known to have ethnic differences; for example, the CYP2D6*10 allele, causing decreased enzyme activity, had a higher frequency in an Asian population (51%) (Johansson *et al*, 1994) than in a white population (2.8%) (Bertilsson and Dahl, 1996). These ethnic differences in the genetic polymorphisms may produce the discrepancy between the results in Murphy *et al* (2003) and those in the present study. However, similar to this study, the subjects in the study by Gerstenberg *et al* were all Japanese patients. In the former study, the patients were divided into three genotype groups by the number of CYP2D6 mutated alleles: *1/*1, *1/*5 or *1/*10, and *5/*10 or *10/*10, whereas, in this study, the patients were divided into two genotype groups by the degree of enzyme activity: *1/*1 or *1/*10 and *10/*10, *1/*5 or *5/*10. We previously reported that one *5 allele had a greater impact on the metabolism of haloperidol, a substrate of CYP2D6, than one *10 allele (Someya *et al*, 2003). Since *1/*10 has only one mutation causing decreased enzyme activity, it was supposed that the enzyme activity of *1/*10 was almost equal to that of the *1/*1 genotype in this study. These differences in analysis may explain the inconsistency between the two studies.

It was demonstrated that a pharmacodynamic factor such as the A-1438G polymorphism of the 5-HT2A receptor gene and a pharmacokinetic factor such as the CYP2D6 gene polymorphism had synergistic effects on the prediction of the gastrointestinal side effects induced by fluvoxamine in Japanese depressed patients. However, since there have been several previous studies that were inconsistent with our results, much research remains to be carried out to explain the discrepancies.

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ORIGINAL ARTICLE

The effect of 5-hydroxytryptamine 3A and 3B receptor genes on nausea induced by paroxetine

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We investigated the effect of 5-hydroxytryptamine 3A and 3B receptor (*HTR3A* and *HTR3B*) gene polymorphisms on nausea induced by paroxetine in Japanese psychiatric patients. Blood samples were collected from 78 individuals after at least 2 weeks treatment with the same daily dose of paroxetine. The patients visited every 2 weeks and the paroxetine dose was changed in response to their clinical symptoms. Nausea was assessed at each visit. The Tyr129Ser polymorphism of the *HTR3B* gene had a significant effect on the incidence of nausea ($P=0.038$). Logistic regression analysis also showed that patients with the Tyr/Tyr genotype had a 3.95-fold ($P=0.048$) higher risk of developing nausea than patients with the Ser allele. *HTR3A* gene polymorphisms and the *CYP2D6* gene polymorphisms had no significant effect on the incidence of nausea. The mean score of nausea severity was corrected by the Bonferroni test. *HTR3B* gene polymorphisms are significant predictors of paroxetine-induced nausea.
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Keywords: HTR3A; HTR3B; paroxetine; nausea; gene polymorphism

Introduction

Nausea is a severe side effect induced by selective serotonin reuptake inhibitors (SSRIs). In general, SSRIs are better tolerated than tricyclic antidepressants, although gastrointestinal side effects can have an incidence of up to 40%, which can be severe enough to lead to early treatment discontinuation.[1,2] Recently, 5-hydroxytryptamine 3 receptors (HTR3) have been considered to have an important role in SSRI-induced gastrointestinal side effects, since HTR3 antagonists cisapride and ondansetron were reported to reduce SSRI-induced gastrointestinal side effects.[3]

HTR3 is a ligand-gated ion channel that mediates fast synaptic neurotransmission.[4] Central and peripheral HTR3 have different structures and different properties.[5] HTR3 exist in the area called the chemoreceptor trigger zone of the medulla oblongata, and are thought to be involved with the vomiting reflex.[6] HTR3 are also distributed in the autonomic, enteric and sensory nervous systems.[7] They also regulate the control pain sensation, movement of the digestive tract and vomiting by prompting nerve depolarization.[8] In particular, modification of HTR3 on the small intestinal mucosa is one of the mechanisms for regulation of antineoplastic-induced nausea and vomiting.[9] We do not have any data about the binding affinity of paroxetine for the 5-HT3 receptors. However, HTR3 antagonists such as cisapride and ondansetron were used for the therapy of nausea induced by SSRIs, and SSRIs such as fluvoxamine display a relatively high affinity for HTR2A. Thus, we considered that there might be some

relationship between nausea induced by SSRIs such as paroxetine and the function of HTR3.

Murphy *et al.*[10] report that the T102C polymorphism of the *HTR2A* gene may predict the treatment discontinuation caused by paroxetine-induced side effects in older patients with depression. *HTR3A* and *3B* genes have been assigned to chromosome 11q23.1–q23.3,[11] and several genetic variations have been reported. Tremblay *et al.*[12] report that variations in the *HTR3B* gene predict the efficacy of antiemetic treatment in cancer patients. However, no previous studies have investigated the effects of *HTR3A* and *3B* gene polymorphisms on the occurrence of SSRI-induced nausea.

Cytochrome P450 (CYP) 2D6 has been shown to be involved in the metabolism of paroxetine, and CYP2D6 is known to have genetic polymorphisms that affect enzyme activity.[13] These observations suggest that the *CYP2D6* gene polymorphism may be a predictor for paroxetine-induced side effects. On the other hand, Gerstenberg *et al.*[14] showed that steady-state plasma concentrations of fluvoxamine are not associated with incidence of nausea, and that *CYP2D6* genotype does not affect nausea development. Therefore, further studies are needed to clarify whether *CYP2D6* gene polymorphisms affect SSRI-induced side effects.

In this study, we investigated the effects of pharmacodynamic factors, such as *HTR3A* and *3B* gene polymorphisms, and the effects of pharmacokinetic factors, such as *CYP2D6* genotype, on the occurrence of paroxetine-induced nausea in Japanese psychiatric patients.

Materials and methods

Subjects

This study was conducted at Niigata University Medical Hospital, Japan, and the study protocol was approved by the Hospital Ethics Committee. Each subject provided written informed consent before enrolment. The subjects comprised 78 Japanese psychiatric outpatients (28 males, 50 females) aged 38.4±13.8 years (mean±s.d., range 18–70 years.). Thirty-nine subjects had major depressive disorder, 25 had anxiety disorders, six had adjustment disorder, seven had a depressive disorder not otherwise specified, and one had other mood disorders. All patients were diagnosed according to DSM-IV-TR. The exclusion criteria were additional diagnoses of Axis I or II of DSM-IV-TR. Demographic data, medical history and laboratory data, including hematology, serology, electrolytes and urine analysis, were collected for each patient. Patients with obvious physical illnesses were excluded from the study. All patients were orally treated with paroxetine for their psychiatric illness. No patients were being treated with antiemetic medication during our study.

Study design

The patients visited the hospital every 2 weeks and side effects, including nausea, were assessed at each visit. The paroxetine dose was increased from 10 or 20 to 30 and 40 mg/day in response to clinical symptoms. We rated the side effects during the last 2 weeks and evaluated the severity of nausea according to our original scale, which included five graded items: 0, no nausea; 1, mild nausea for less than during the last 2 weeks; 2, mild nausea for more than 1 week during the last 2 weeks; 3, continuous, moderate nausea during the last 2 weeks; 4, continuous, severe nausea and vomiting during the last 2 weeks. Subjects with a score of 0 or 1 were defined as subjects without nausea, and those with a score or 2, 3 or 4 were defined as having nausea. The side effect raters were blind to the patients' genotypes.

Blood sampling

Blood sampling was performed using a Venoject® tube containing EDTA-Na (Terumo Japan, Tokyo, Japan) at week 1 for genotype detection and subsequently at the first appearance of nausea to measure the concentration of paroxetine. Blood samples were taken at 12 h after the final ingestion of paroxetine. Venous blood, 7 ml, were collected, and genomic DNA was extracted from the peripheral leukocytes by utilizing a QIAamp Blood Kit (QIAGEN, Valencia, CA, USA) within 2 h of collection.

Genotyping and determination of plasma concentration

Polymerase chain reaction (PCR) was used to determine the C195T and Pro16Ser genotypes of *HTR3A* gene according to the method of Niesler *et al.*[15] and the Tyr129Ser genotype of *HTR3B* gene according to the method of Tremblay *et al.*[12] *CYP2D6*10* alleles causing decreased enzyme activity were identified by the C188T mutation using a two-step PCR as described by Johansson *et al.*[16] A long-PCR analysis was used to detect the **5* allele causing a lack of enzyme activity as described by Steen *et al.*[17]

The plasma concentration of paroxetine was measured using column-switching high-performance liquid chromatography (HPLC) with ultraviolet detection. Paroxetine was extracted from plasma, to which cisapride had been added as an internal standard, with hexane–chloroform, and the extract was subjected to automated column-switching HPLC using a TSK BSA-C8 precolumn (Tosoh, Tokyo, Japan) for sample clean-up, and a TSK gel ODS-80TS column (Tosoh) for separation.

Statistical analysis

Statistical analysis was performed using SPSSII for Windows. Genotype and allele distributions were analyzed by the χ^2 test. The clinical and demographic characteristics, sex, age, daily dose and paroxetine concentration were compared among groups by the unpaired *t*-test. The mean score of nausea severity was compared among each genotype group by one-way analysis of variance, and *post hoc* analysis of the mean score of nausea severity was carried out by using Bonferroni's test. Logistic regression analysis was used to compare the probability of the incidence of nausea. The level of significance was set at $P<0.05$.

Results

The genotype frequencies of the *HTR3A*, *HTR3B* and *CYP2D6* genes are shown in Tables 1–3. All of these genetic variations were in Hardy–Weinberg equilibrium. *HTR3A* C195T polymorphism genotype was not detected in four patients. There were no differences in nausea between each diagnostic groups (e.g. major depressive disorders and anxiety disorders ($P=0.12$)). Therefore, we consider that the nausea observed in the present study was induced only by paroxetine. There were also no differences in *HTR3A*, *HTR3B* and *CYP2D6* polymorphisms between major depressive disorders and anxiety disorders ($P=0.341$).

Effects of HTR3A *and* HTR3B *gene polymorphisms*

The genotype distribution of *HTR3A* and *HTR3B* gene polymorphisms are shown in Tables 1 and 2. No significant differences were demonstrated for sex, age, and paroxetine daily dose and plasma concentration among each genotype group. Association analysis revealed that genotype frequencies of *HTR3A* Pro16Ser and *HTR3B* C195T polymorphisms did not significantly differ between subjects with and without nausea (Pro16Ser genotype: $\chi^2=0.912$, $df=2$, $P=0.634$; C195T genotype: $\chi^2=2.128$, $df=2$, $P=0.546$). There was a significant difference in genotypic distribution associated with *HTR3B* Tyr129Ser polymorphism between patients with and without nausea ($\chi^2=6.547$, $df=2$,

Table 1 Genotypic distribution and demographic data of the *HTR3A* Pro16Ser and C195T

	HTR3A Pro16Ser			*HTR3A C195T*		
	Pro/Pro	*Pro/Ser*	*Ser/Ser*	*C/C*	*C/T*	*T/T*
Sex (M/F)	23/34	5/13	0/3	15/22	11/16	1/9
P		*0.261*			*0.291*	
Age (±s.d.)	37.8 (13.8)	38.2 (14.2)	52.0 (4.4)	37.8 (13.8)	38.2 (14.2)	52.0 (4.4)
P		*0.411*			*0.370*	
Daily dose of PRX (±s.d.)	23.0 (10.5)	20.0 (9.7)	20.0 (17.3)	20.5 (9.7)	25.9 (10.5)	19.0 (12.9)
P		*0.408*			*0.205*	
Concentration of PRX (±s.d.)	66.4 (89.3)	28.9 (31.3)	22.3 (29.8)	42.8 (61.1)	65.8 (90.6)	52.9 (80.6)
P		*0.167*			*0.707*	
Nausea (+) ($n=15$)	12	3	0	9	5	1
%	(21.1%)	(16.7%)	(0.0%)	(24.3%)	(18.5%)	(10.0%)
Nausea (−) ($n=63$)	45	15	3	28	22	9
%	(78.9%)	(83.3%)	(100.0%)	(75.7%)	(81.5%)	(90.0%)
P		*0.634*			*0.546*	
Mean score of severity of nausea	0.21 (0.411)	0.17 (0.383)	0 (0)	0.1 (0.316)	0.19(0.396)	0.24(0.435)
P		*0.643*			*0.593*	

Table 2 Genotypic distribution and demographic data of the *HTR3B* Tyr129Ser

	HTR3B Tyr129Ser			
	Tyr/Tyr	*Tyr/Ser*	*Ser/Ser*	*Tyr/Ser+Ser/Ser*
Sex (M/F)	16/19	10/26	2/5	12/31
P		*0.264*		*0.103*
Age (±s.d.)	36.3 (11.5)	38.9 (15.1)	46.1 (16.6)	40.1 (15.4)
P		*0.099*		*0.267*
Daily dose of PRX (±s.d.)	21.4 (10.6)	21.9 (10.6)	27.1 (9.5)	22.8 (10.5)
P		*0.4*		*0.939*
Concentration of PRX (±s.d.)	37.6 (46.9)	66.4 (100.2)	95.0 (77.2)	71.1 (96.6)
P		*0.124*		*0.064*
Nausea (+) ($n=15$)	11	4	0	4
(%)	(31.4%)	(11.1%)	(0.0%)	(9.3%)
Nausea (−) ($n=63$)	24	32	7	39
(%)	(68.6%)	(88.9%)	(100.0%)	(90.7%)
P		*0.038**		*0.014**
Mean score of severity of nausea	0.54 (0.919)	0.14 (0.487)	0 (0)	0.12 (0.448)
P		*0.030***		*0.009***

*$P<0.05$, significant difference from patients with nausea (χ^2 text).
**$P<0.05$, significant difference among the three genotypes (one-way analysis of variance).

Table 3 Genotypic distribution and demographic data of *CYP2D6* genotype groups

	CYP2D6		
	**1/*1*	**1/*5, *1/10*	**5/*10, *10/*10*
Sex (M/F)	17/34	5/7	6/9
P		*0.807*	
Age (±s.d.)	38.6 (14.9)	39.7 (10.7)	36.7 (12.9)
P		*0.126*	
Daily dose of PRX (±s.d.)	21.4 (10.2)	24.2 (11.7)	23.3 (11.1)
P		*0.925*	
Concentration of PRX (±s.d.)	50.7 (76.3)	89.5 (114.4)	47.5 (51.2)
P		*0.287*	
Nausea (+) ($n=15$)	9	2	4
(%)	(17.6)	(16.7)	(26.7)
Nausea (−) ($n=63$)	42	10	11
(%)	(82.4)	(83.3)	(73.3)
P		*0.716*	
Mean score of severity of nausea	0.18 (0.385)	0.17 (0.389)	0.27 (0.458)
P		*0.725*	

Table 4 Logistic regression analysis of independent valiables to Nausea

Independent valiable	*Partial regression coefficients*	P	*Odd ratio (95% confidence interval)*
Sex	−0.040	0.111	1.115 (0.518–3.225)
Age	−0.793	0.041	1.200 (0.810–1.110)
Daily dose of Paroxetine	−0.538	0.320	0.447 (0.112–3.154)
HTR3B Tyr129Ser genotype	−0.148	0.048*	3.950 (1.009–15.455)

*$P<0.05$, statistical significance.

$P=0.038$). The proportion of Ser allele carriers (i.e., patients with either Tyr/Ser or Ser/Ser) was significantly higher in the group without nausea ($\chi^2=6.082$, $df=1$, $P=0.014$). There were significant differences in the severity score of nausea among the three genotypes (score: 0.54±0.91, 0.14±0.49 and 0±0, $df=2$, $P=0.03$).

The results of logistic regression analysis are shown in Table 4. The incidence with or without nausea was used in the analysis as an independent variable, and sex, age, daily dose of paroxetine and the genotypes of *HTR3B* Tyr129Ser were added as potential confounders. This analysis also showed that there was a significant association between nausea and *HTR3B* Tyr129Ser genotype. ($P=0.048$; $OR=3.95$; 95% $CI=1.009$–15.455).

Effect of CYP2D6 gene polymorphism

Five CYP2D6 genotypes were identified: **1/*1* ($n=51$), **1/*5* ($n=1$), **1/*10* ($n=11$), **5/*10* ($n=3$) and **10/*10* ($n=12$). The allele frequencies of the **5* and **10* alleles were 2.6 and 24.4%, respectively. Patients were divided into three genotype groups according to the number of mutated alleles: 51 patients with the **1/*1* genotype, 12 with the **1/*10* and **1/*5* genotypes and 15 with the **5/*10* and **10/*10* genotypes. No significant differences were demonstrated for sex, age, and paroxetine daily dose and plasma concentration between those three genotype groups (Table 3).

There were no significant differences in the incidence of nausea between the three genotype groups ($\chi^2=1.029$, $df=2$, $P=0.716$). We also divided patients into two genotype groups: 61 patients with the **1/*1* or **1/*10* genotype were termed normal metabolizers, and 17 patients with the **10/*10*, **1/*5* or **5/*10* genotype were termed low metabolizers. However, there were also no significant differences in the incidence of nausea between the two genotype groups.

Discussion

We screened for two polymorphisms in the *HTR3A* gene and one variant in the *HTR3B* gene as a pharmacodynamic factor, and *CYP2D6* gene polymorphisms (**1*, **5*, **10* alleles) as a pharmacokinetic factor. To our knowledge, the present study is the first demonstration that the *HTR3B* gene may predict the incidence of paroxetine-induced nausea in Japanese psychiatric patients.

Kaiser *et al.*[18] reported that polymorphisms of the *HTR3A* gene may not serve as pharmacogenetic predictors of antiemetic treatment with HTR3 antagonists in cancer