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

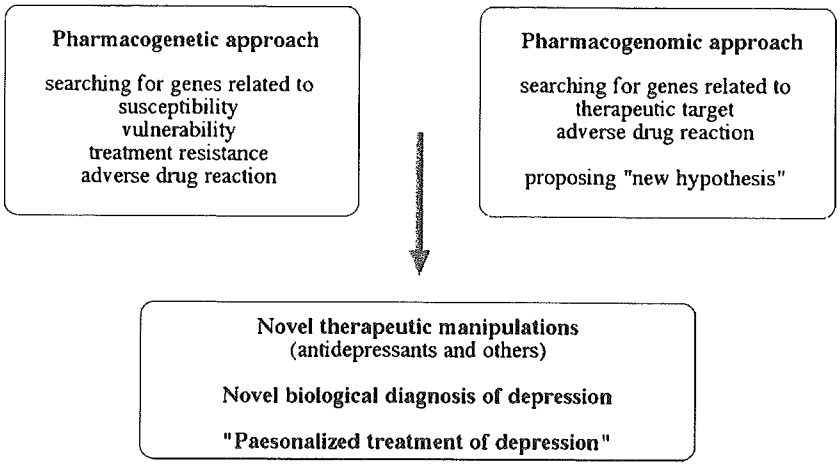

Fig. 5. Pharmacogenetics and pharmacogenomics in depression research.

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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Original Papers

Polymorphisms in the 5-hydroxytryptamine 2A receptor and cytochromeP4502D6 genes synergistically predict fluvoxamine-induced side effects in Japanese depressed patients

Running title: Pharmacogenetics of SSRI-induced side effects

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ABSTRACT

5-hydroxytryptamine (5-HT) receptors are thought to be associated with the gastrointestinal side effects induced by selective serotonin reuptake inhibitors. CytochromeP450 (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 endpoint 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 (NMs) 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.

Key words: cytochrome P-450 2D6, fluvoxamine, side effects, 5-HT2A, genetic polymorphism

Introduction

Fluvoxamine is widely available in Europe and the United States, and was introduced into clinical use as the first approved selective serotonin (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, stomachache 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 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. CytochromeP450 (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 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 males, 53 females) aged 40.5±15.7 years (mean age ± S.D.). Eighty-five subjects had DSM-IV diagnoses of major depressive disorder, 7 had adjustment disorder with depressed mood, 6 had a depressive disorder not otherwise specified and 2 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 5 items; nausea, vomiting, anorexia, diarrhea and stomachache. Because 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 hours after the final ingestion of fluvoxamine. Seven milliliters 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 hours 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). CYP*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-Meyer 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 7 patients. The other 3 genotypes of 4 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, 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% confidence

interval, 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 (χ^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).

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 (Table 2, 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 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 (χ^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).

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