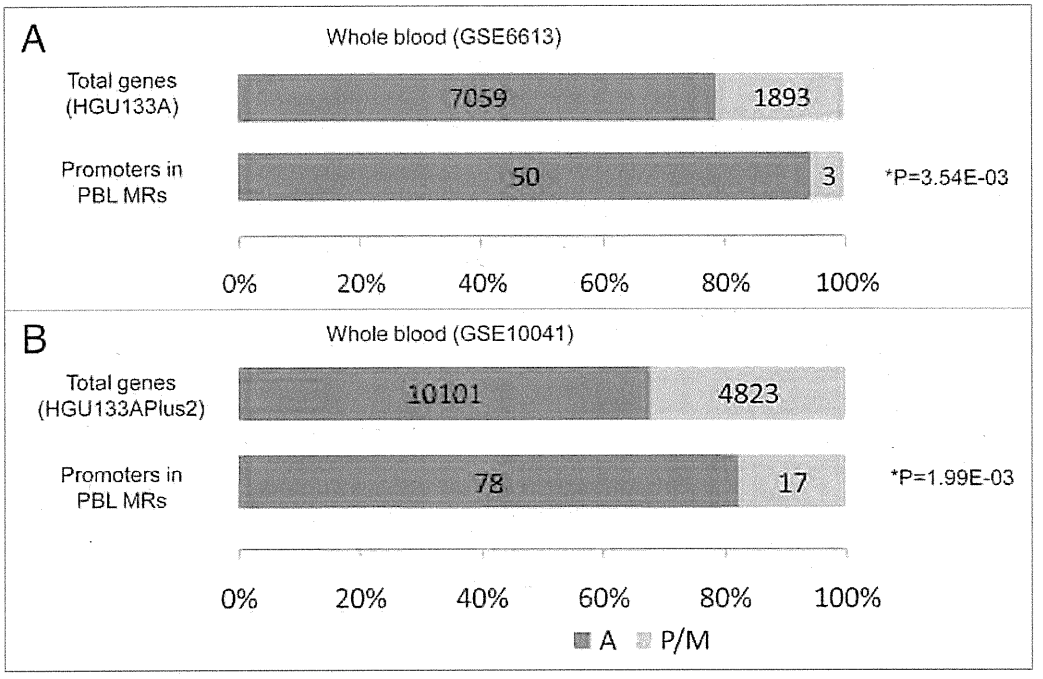

Figure 4. Effect of methylation in promoter regions overlapping with CpG islands on gene expression in LCLs. The numbers of genes with containing at least one probe with present or marginal flags, as well as that of genes whose all probes were called as absent, are indicated. If all samples across the chosen study showed absent flags with respect to a certain probe set, we considered this "absent" and "present" probes were determined similarly. If one gene had both probe sets of present/marginal and absent, the gene was classified as an expressed gene. (A) HGU133plus2.0 platform data set from GEO (GSE12408 (N = 17)[16]), (B) HGU133plus2.0 platform data set from GEO (GSE13122 (N = 13)[17]). Fisher's exact test p values are given. A: genes with absent probes, P/M: genes with present or marginal probes, N = number of control samples.

Table 3. The number of specifically methylated regions in individual subjects

	C1 (29 M)	C2 (25 F)	C3 (25 F)	C4 (52 M)	Number of MRs overlapping among four cell lines
PBL-specific MRs	6 (0**/0*)	137 (0**/9*)	29 (0**/5*)	94 (0**/8*)	0
LCL-specific MRs	902 (11**/189*)	194 (9**/50*)	496 (11**/167*)	35 (3**/12*)	1

PBL, peripheral blood lymphocyte; LCL, lymphoblastoid cell line; MRs, methylated regions; *number of MRs overlapping in other one cell line. **number of MRs overlapping in other two cell lines.

Committee of RIKEN. Informed consent was obtained from all participants.

Sample preparation. LCLs were previously established by transforming lymphocytes with EB virus using standard techniques.[20] Briefly, lymphocytes were separated from peripheral blood and cultured with RPMI 1640 medium containing 20% fetal bovine serum (GIBCO, Carlsbad, CA), penicillin and streptomycin (50 μg/ml each) and supernatant of the B95-8 cell culture infected with EB virus. The cells were passaged every week until stable growth was exhibited. Thereafter, the cells were passaged two or three times a week using similar medium, except for the addition of 10% fetal bovine serum. DNA was extracted using standard protocols.

Luminometric methylation assay. The LUMA was performed as initially described by Karimi et al.[10,11] with slight modifications. Each genomic DNA (200 ng) was digested with *Eco*RI (New England Biolabs, Beverly, MA) in two separate 20 μl reactions containing 20 mM Tris-acetate, 10 mM Mg-acetate, 50 mM K-acetate pH 7.9, 1 mM dithiothreitol and 10 U restriction enzyme. After incubation at 37°C for 4 h, one of the digests was cleaved with 15 U *Hpa*II (New England Biolabs) and the other digest was cleaved with 15 U *Msp*I (New England Biolabs) at 37°C overnight. Then, 20 μl of annealing buffer (20 mM Tris-acetate, 2 mM Mg-acetate pH 7.6) was added to the cleavage reactions, and the samples were placed in a PSQ96™ MA system (Biotage AB, Uppsala, Sweden). Using PyroMark Gold Q96 Reagents for pyrosequencing (QIAGEN, Crawley UK), the instrument was programmed to add dNTPs in four consecutive steps including Step 1: dATPαS (the derivative of dATP, used because it does not directly react with luciferase and prevents non-specific signals); Step 2: a mixture of dGTP + dCTP; Step 3: dTTP and Step 4: a mixture of dGTP + dCTP. Peak heights were calculated using the PSQ96™MA software. The *Hpa*II/*Eco*RI and *Msp*I/*Eco*RI ratios were calculated as (dGTP + dCTP)/dATP for the respective reactions. The *Hpa*II/*Msp*I ratio was defined as (*Hpa*II/*Eco*RI)/(*Msp*I/*Eco*RI).

Using LCL DNA derived from a Japanese male, we prepared unmethylated DNA by whole genome amplification using a Genomiphi DNA Amplification kit (GE Healthcare Life Science). Methylated DNA was then obtained by treating unmethylated

DNA with SssI methylase (New England Biolabs). Methylated and unmethylated DNAs were mixed in different proportions to obtain samples with 0, 25, 50, 75 or 100% methylated DNA and used as standard samples for the LUMA.

Each assay was replicated, and the results were plotted as mean value ± SEM for a given concentration.

Enrichment of methylated DNA. We used a MethylCollector Ultra Kit (Active Motif, Carlsbad, CA) to enrich the methylated DNA according to the manufacturer's protocol. In this method, His-tagged recombinant MBD2b and its binding partner MBD3L1 were combined together to increase the affinity of MBD2b for CpG-methylated DNA. The sensitivity is such that it can enrich methylated DNA fragments with as few as five methylated CpG sites. A total of 100 ng DNA fragments, which were sonicated to produce 100–300 bp fragments using a sonicator (Covaris, Inc., Woburn, MA) were incubated with a His-tagged recombinant MBD2b/MBD3L1 protein complex. These protein-DNA complexes were captured with nickel-coated magnetic beads. After stringent washing, methylated DNA was eluted. Eluted DNA was further purified using the MiniElute PCR purification kit (QIAGEN).

Promoter tiling array. We used a GeneChip Human Promoter 1.0R array (Affymetrix, Santa Clara, CA), which contains 4.6 million probes tiled to cover over 25,500 human promoter regions. The probes are 25 bp long and are spaced 35 bp from the middle of each probe to the next, leaving a gap of approximately 10 bp between probes. Sample preparation for the tiling array using the eluted methylated DNA fraction was performed according to the Affymetrix chromatin immunoprecipitation assay protocol provided by the manufacturer (Affymetrix). Briefly, after amplification, DNA samples were labeled using a GeneChip WT Double-Stranded DNA Terminal Labeling Kit (Affymetrix). The samples were hybridized with Affymetrix GeneChip Human Promoter 1.0R arrays. The arrays were stained and washed with GeneChip Fluidics Station 450 and scanned with the GeneChip 3000 7G Scanner. We prepared fully CpG unmethylated DNA obtained by whole genome amplification and used this DNA as a reference sample. The reference sample was also prepared using a Methyl Collector Ultra kit, and the promoter array analyses were replicated.

Tiling array data analysis. *Calculation of correlation coefficients (R).* We used Affymetrix Tiling Array Software to obtain signal intensities of individual probes. Pearson's correlation coefficients between samples were calculated using R script (www.r-project.org).

Detection of methylated regions. MRs were detected using model-based analysis of tiling-arrays (MAT) software.[12] In MAT, probe sequence and copy number on each array is considered for the standardization of the probe value. This can eliminate the need for normalization. Using MAT, we can score enrichment regions with robust p value, and detect enrichment regions from single samples, multiple samples or multiple samples with controls.[12] For all comparisons, we used MAT with a p value of 1e-4, and all other parameters were the defaults. We performed both group and individual analyses with R script using the following steps. Scripts are available upon request.

In the group analysis, we first compared all PBL (N = 4) and LCL (N = 4) samples to a reference sample (i.e., unmethylated DNA) respectively to detect the regions showing the significantly methylated signal compared with a reference sample. These MRs were named PBL MRs and LCL MRs. Only those regions containing six or more CpG sites were further selected because the MethylCollector can collect the DNA fragments that contain five or more CpG sites. We then directly compared all PBL samples to all LCL samples to detect the regions showing significant differences between PBLs and LCLs. These MRs were named PBL-dominant MRs and LCL-dominant MRs. Among the PBL-dominant MRs, the regions overlapping with PBL MRs but not LCL MRs were selected. The selected regions were named PBL-specific MRs. LCL-specific MRs were determined vice versa. These steps highlighted the methylation difference between PBLs and LCLs, and eliminated significant but subtle differences to maximize the chance for successful confirmation by other methods.

In the individual analysis, we compared the PBL or the LCL sample to a reference sample separately to identify PBL MRs or LCL MRs in each of the four subjects, and only those regions containing six or more CpG sites were further selected. We then identified PBL-dominant MRs and LCL-dominant MRs from the direct comparison of the PBLs and LCLs from the same individual. To identify PBL-specific MRs, the regions overlapping with PBL MRs but not LCL MRs were selected among the PBL-dominant MRs. These steps were performed separately for each subject. The PBL-specific MRs were similarly determined. We further identified the number of each PBL- or LCL-specific MRs overlapping among all four, three or two cell lines.

Bisulfite sequencing. Each 500 ng of genomic DNA was converted for methylation sequencing using Epitect bisulfite kits (Qiagen), according to the manufacturer's standard protocol. Completion of sodium bisulfite conversion was confirmed during sequencing by ensuring that known lone cytosines were read as thymines.

Primer pairs were determined using MethPrimer software.[21] PCR products were digested with Thermostable β-Agarase (NIPPON GENE CO., LTD., Tokyo, Japan) and cloned using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA). Single bacterial colonies were subjected to sequencing analysis. Each bisulfite sequencing data was quantified by quantification tool for methylation analysis.[22]

Gene ontology analysis. We collected the genes located within 3 kb of the MRs using the Build 36.1 finished human genome assembly (hg18, Mar 2006) with R script. Ontology analysis was performed using the PANTHER classification system.[23,24] The significantly enriched terms were identified using the Bonferroni correction for multiple comparisons with a cutoff of $p < 0.05$.

Public expression data analysis. We examined the correlation between MRs overlapping with CpG islands in promoter regions and the gene expression status using a public database. We searched PBL expression datasets from the Gene Expression Omnibus (GEO) using the terms "*Homo sapiens*, whole blood and Affymetrix" and selected the two datasets with a large number of control samples (GSE6613[14] and GSE10041,[15]). In the

same way, we searched LCL expression datasets using the terms "*Homo sapiens*, lymphoblastoid cell and Affymetrix" and selected two datasets (GSE12408[16] and GSE13122,[17]). In each dataset, only control subjects were used for data analysis.

First, we examined the expression status of all genes (probe sets). The number of genes with one or more transcripts with present or marginal flag was counted. The number of genes whose all probes were called as absent was also counted. Next, we examined the expression status of the genes with promoter regions (within 10 kb of exon 1) on the PBL or LCL_MRs overlapping with CpG islands.

For statistical analysis, Fisher's exact test was used.

Conclusion

In summary, we performed a comprehensive DNA methylation analysis using both PBL and LCL genomic DNA derived from the same individuals. Our findings indicate that the methylation patterns of promoter regions were largely conserved between PBLs and LCLs. In addition, methylation in promoter regions overlapping with CpG islands was associated with gene silencing in LCLs similarly to PBLs. However, there are some methylation differences between PBLs and LCLs and hypermethylation might be more predominant than hypomethylation in LCLs. LCLs should be used with caution for DNA methylation analysis associated with human diseases, as the methylation patterns of promoter regions in LCLs is not always the same as in PBLs.

Acknowledgements

We are grateful to the Research Resource Center of our institute for performing the microarray analysis.

Note

Supplemental materials can be found at:
www.landesbioscience.com/journals/epigenetics/article/14876

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Progress in Neuro-Psychopharmacology & Biological Psychiatry 34 (2010) 1306–1316

Contents lists available at ScienceDirect

Progress in Neuro-Psychopharmacology & Biological Psychiatry

journal homepage: www.elsevier.com/locate/pnp

Inhibitory effects of SSRIs on IFN-γ induced microglial activation through the regulation of intracellular calcium

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ARTICLE INFO

Article history:
Received 1 February 2010
Received in revised form 29 June 2010
Accepted 14 July 2010
Available online 21 July 2010

Keywords:
Antidepressants
Cytokine
Intracellular Ca^{2+}regulation
Microglia
Nitric oxide

ABSTRACT

Microglia, which are a major glial component of the central nervous system (CNS), have recently been suggested to mediate neuroinflammation through the release of pro-inflammatory cytokines and nitric oxide (NO). Microglia are also known to play a critical role as resident immunocompetent and phagocytic cells in the CNS. Immunological dysfunction has recently been demonstrated to be associated with the pathophysiology of depression. However, to date there have only been a few studies on the relationship between microglia and depression. We therefore investigated if antidepressants can inhibit microglial activation in vitro. Our results showed that the selective serotonin reuptake inhibitors (SSRIs) paroxetine and sertraline significantly inhibited the generation of NO and tumor necrosis factor (TNF)-α from interferon (IFN)-γ-activated 6-3 microglia. We further investigated the intracellular signaling mechanism underlying NO and TNF-α release from IFN-γ-activated 6-3 microglia. Our results suggest that paroxetine and sertraline may inhibit microglial activation through inhibition of IFN-γ-induced elevation of intracellular Ca^{2+}. Our results suggest that the inhibitory effect of paroxetine and sertraline on microglial activation may not be a prerequisite for antidepressant function, but an additional beneficial effect.

1. Introduction

Accumulating data suggest the importance of immunological dysfunction in the pathophysiology of depression (Anisman 2009; Miller et al., 2009; Muller and Schwarz 2007; Raison et al., 2006; Schiepers et al., 2005; Dowlati et al., 2009). Microglia are generally considered to be the immune cells of the CNS and they respond to any kind of pathology with a reaction termed microglial activation (Hanisch and Kettenmann, 2007). Microglial activation plays an important role in the pathophysiology of neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD) via the release of pro-inflammatory cytokines, nitric oxide (NO) or reactive oxygen species (Inoue and Tsuda, 2009, McGeer et al., 1993; Stoll and Jander 1999). Furthermore, an elevated microglial density has been observed in patients with depression or schizophrenia who have committed suicide (Steiner et al., 2008).

It has been suggested that impaired hippocampal neurogenesis contributes to the pathogenesis of depression and it has been demonstrated that the neuroinflammation associated with microglial activation inhibits hippocampal neurogenesis (Duman 2004; Ekdahl et al., 2003; Monje et al., 2003). These studies have further shown that indomethacin, a conventional non-steroidal anti-inflammatory drug, and minocycline, which inhibit microglial activation, can restore this impaired neurogenesis (Ekdahl et al., 2003; Monje et al., 2003). Pro-inflammatory cytokines such as interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) have been reported to be negative regulators of hippocampal neurogenesis (Monje et al., 2003; Vallieres et al., 2002; Iosif et al., 2006; Kaneko et al., 2006; Koo and Duman 2008). It has also been reported that NO inhibits neurogenesis while nitric oxide synthase (NOS) inhibitors show antidepressant-like activity (Zhou et al., 2007; Joca and Guimaraes, 2006). Antidepressant treatment increases neurogenesis in adult hippocampus (Duman 2004; Malberg et al., 2000; Santarelli et al., 2003) and the behavioral effects of antidepressants may, in part, be due to stimulation of such hippocampal neurogenesis (Santarelli et al., 2003).

In animal experiments, chronic administration of pro-inflammatory cytokines has been shown to induce symptoms similar to depression. These symptoms are referred to as sickness behavior and include loss of

Abbreviations: IFN-γ, interferon-γ; LPS, lipopolysaccharide; NO, nitric oxide; TNF-α, tumor necrosis factor-α; $[Ca^{2+}]i$, intracellular Ca^{2+} concentration; 5-HT, 5-hydroxytryptamine (serotonin); SSRI, selective serotonin reuptake inhibitor; 5-HTT, 5-hydroxytryptamine (serotonin) transporters; JAK-STAT, Janus kinase-signal transducer and activator of transcription; DMSO, dimethyl sulfoxide; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetra-zolium bromideicroglia; PBS, phosphate buffered saline; GM-CSF, granulocyte macrophage-colony stimulating factor MAPK, mitogen-activated protein kinase; ERK, extracellular signal regulated kinase; PKC, protein kinase C; BIM I, bisindolylmaleimide I.

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doi:10.1016/j.pnpbp.2010.07.015

appetite, insomnia and lack of interest (Dantzer et al., 2008). Pro-inflammatory cytokines such as TNF-α and interferon (IFN)-γ activate the tryptophan- and serotonin-degrading enzyme indoleamine 2, 3-dioxygenase (IDO). The increased consumption of serotonin and its precursor tryptophan by activated IDO leads to a reduced availability of serotonin for serotonergic transmission (Muller and Schwarz, 2007). Furthermore, pro-inflammatory cytokines such as IL-1β and TNF-α also reduce serotonergic transmission through the activation of serotonin transporters (5-HTT) (Zhu et al., 2006).

The above data encourage speculation that antidepressants play an important role in the pathophysiology of depression through inhibitory effects on microglial activation. To our knowledge, only a few recent studies have shown that some antidepressants inhibited the production of NO and/or pro-inflammatory cytokines from activated microglia in vitro (Hashioka et al., 2007; Hwang et al., 2008; Lim et al., 2009; Obuchowicz et al., 2006; Vollmar et al., 2008).

In the present study, we demonstrate that antidepressants such as paroxetine and sertraline, that are typical selective serotonin reuptake inhibitors (SSRIs), also have an inhibitory effect on the release of NO and TNF-α from IFN-γ-induced microglial cells.

Some SSRIs have been reported to induce apoptosis through an effect on intracellular Ca^{2+} regulation in Burkitt lymphoma and human osteosarcoma cells (Serafeim et al., 2003; Chou et al., 2007). We have also recently reported that aripiprazole, which is used to treat depression, inhibited microglial activation through suppression of IFN-γ-induced elevation of intracellular Ca^{2+} in microglia (Kato et al., 2008). Therefore, we also investigated the effect of paroxetine and sertraline on intracellular Ca^{2+} regulation of microglia. We also investigated whether bupropion, and agomelatine that have much lower effect on 5-HTT than paroxetine and sertraline, have similar effects on microglia. Although antidepressant effect of agomelatine is debatable, agomelatine is reported to be an antidepressant at doses of 25–50 mg/day (Dubovsky and Warren 2009).

2. Methods

2.1. Materials

All experimental procedures were conducted in accordance with the Standard Guidelines for Animal Experiments of the Graduate School of Medicine, Kyushu University.

Sertraline was kindly gifted by Pfizer Inc. (New York, NY, USA). Paroxetine was purchased from Toronto Research Chemicals Inc. (North York, Canada). The JAK inhibitor 1 was purchased from Calbiochem (San Diego, CA, USA). Agomelatine, bupropion, serotonin, and all other main chemicals were purchased from Sigma (St. Louis, MO, USA). Recombinant mouse granulocyte macrophage-colony stimulating factor (GM-CSF) and recombinant IFN-γ were purchased from R&D systems (Minneapolis, MN, USA). Paroxetine and bupropion were dissolved in phosphate buffered saline (150 mM NaCl, 5 mM phosphate, pH 7.4). The final concentration of paroxetine was 0.5–5 μM and the final concentration of bupropion was 1–10 μM. Sertraline and agomelatine were initially dissolved at a concentration of 20 mM in dimethyl sulfoxide (DMSO) and were then diluted to 2 mM with phosphate buffered saline (PBS). The final concentration of sertraline was 0.5–5 μM and the final concentration of agomelatine was 1–10 μM. DMSO, at the highest concentration (0.05%), was not toxic to the cells under the experimental conditions used.

2.3. Cell cultures

The murine microglial cell line, 6-3, was kindly gifted by Prof. M. Sawada of Nagoya University. The 6-3 cells were established from neonatal C57BL/6 J (H-2b) mice using a non-enzymatic and non-viral-transformation procedure and closely resemble primary cultured microglia (Kanzawa et al., 2000). The 6-3 cells were cultured in Eagle's minimal essential medium containing 0.3% $NaHCO_3$, 2 mM glutamine, 0.2% glucose, 10 g/mL insulin and 10% fetal bovine serum, and were maintained at 37 °C in a 5% CO_2 and 95% air atmosphere. The culture medium was supplemented with 1 ng/mL of recombinant mouse GM-CSF since this was required for proliferation of the 6-3 cells (Kanzawa et al., 2000). The culture medium was renewed twice per week.

Primary mixed cells were prepared from the whole brain of postnatal day three Sprague–Dawley rats (n = 6) as described previously (Kato et al., 2008). The dissected tissue was dissociated into single cells using a Cell Strainer (BD Falcon, Franklin Lakes, NJ, USA), and the dissociated cells were suspended in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum (FBS), (10% FBS/DMEM). The cells were incubated at 37 °C in an incubator with a 5% CO_2 and 95% air atmosphere. Microglia cells were selected by adhesion to an Aclar plastic film (Nisshin EM, Tokyo, Japan) for 2 h in 10% FBS/DMEM. The films were briefly rinsed with PBS, transferred to fresh 10% FBS/DMEM, and the harvested cells were expanded for 5 days (Kato et al., 2008). The purity of the isolated microglia was assessed by immunocytochemical staining of the microglial marker Iba-1 which positively stained >99% of the cells.

2.3. Assay of nitrite production

The accumulation of NO_2^-, a stable end-product that is extensively used as an indicator of NO production by cultured cells, was assayed using the Griess reaction. The 6-3 microglial cells were plated on 96-well tissue culture plates at a density of 1×10^5 cells per 200 μL per well and were then pre-incubated in the presence or absence of each antidepressant (paroxetine, sertraline, agomelatine, or bupropion) for 24 h followed by incubation in the presence or absence of 50 U/mL IFN-γ at 37 °C for a further 24 h. The cell-free supernatants were then mixed with equal amounts of the Griess reagent. Samples were incubated at room temperature for 15 min and the absorbance was then read at 540 nm using a plate reader (Labsystems Multiscan MS, Frankfurt, Germany). Kits for carrying out the Griess assay were purchased from Dojindo Molecular Technologies (Kumamoto, Japan). Rat primary microglial cells were plated on 96-well tissue culture plates at a density of 5×10^3 cells per 200 μL per well. The cells were pre-incubated in the presence or absence of the antidepressant paroxetine or sertraline for 24 h followed by incubation in the presence or absence of LPS (50 ng/mL or 1 μg/mL) at 37 °C for a further 24 h. The collected media were then assayed for NO accumulation as described above.

2.4. Assay of TNF-α release

The 6-3 cells were plated on 96-well tissue culture plates at a density of 1×10^5 per 200 μL per well. The cells were pre-incubated in the presence or absence of paroxetine or sertraline for 24 h followed by incubation in the presence or absence of 50 U/mL IFN-γ at 37 °C for a further 24 h. The collected media were then assayed for TNF-α accumulation. TNF-α-release into the culture medium was measured using a mouse TNF-α enzyme-linked immunosorbent assay (ELISA) kit based on the quantitative "sandwich" enzyme immunosorbent technique. The assay was carried out according to the manufacturer's protocol (Biosource International, Camarillo, CA, USA). The sensitivity of this assay was 4 pg/mL. Rat primary microglial cells were plated on 96-well tissue culture plates at a density of 5×10^3 cells per 200 μL per well. The cells were pre-incubated in the presence or absence of the antidepressant paroxetine or sertraline for 24 h followed by incubation in the presence or absence of 1 μg/mL LPS at 37 °C for a further 24 h. The collected media were then assayed for TNF-α accumulation as described above. The assay was carried out according to the manufacturer's protocol (R&D systems, Minneapolis, MN, USA).

2.5. Assay of IL-4 release

The 6-3 cells were plated on 96-well tissue culture plates at a density of 1×10^5 per 200 μL per well. The cells were treated in the presence or absence of paroxetine, sertraline or IFN-γ (50 U/mL). After 24 h incubation at 37 °C, the collected media were then assayed for IL-4 accumulation. IL-4-release into the culture medium was measured using a mouse IL-4 ELISA kit. The assay was carried out according to the manufacturer's protocol (Thermo Scientific, IL, USA). The sensitivity of this assay was 5 pg/mL.

2.6. Intracellular Ca^{2+} imaging

Intracellular Ca^{2+} imaging was performed in HEPES buffer (150 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose and 10 mM HEPES, pH 7.4 with Tris–OH) at room temperature. Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was monitored using the calcium-sensitive fluorescent indicator fura-2 acetoxymethyl ester (AM) (Mizoguchi et al., 2002; Grynkiewicz et al., 1985). The 6-3 cells plated on glass-base dish (Iwaki, Tokyo, Japan) and cultured for 24 h, were loaded with 5 μM fura-2 AM (Dojindo, Wako City, Japan), and a membrane permeable Ca^{2+} indicator dye for 20 min at room temperature and were washed three times with HEPES buffer before measurement. External HEPES buffer was constantly perfused (10 mL/min) during measurements that were carried out using an inverted microscope (20×; Olympus IX70-22FL, Olympus Co. Tokyo, Japan). For fura-2 excitation, the cells were illuminated with two alternating wavelengths of 340 and 380 nm using a computerized system for a rapid dual wavelength Xenon arc. The emitted light was collected at 510 nm using a cooled CCD camera (C4742-95ER, Hamamatsu Photonics, Hamamatsu, Japan) and images were stored every 5 s. These series of sequential data were analyzed using the AquaCosmos software package (Hamamatsu photonics, Hamamatsu, Japan). The $[Ca^{2+}]_i$ was calculated from the ratio (R) of the fluorescence recorded at 340 and 380 nm excitation wavelengths for each pixel within a cell boundary (AquaCosmos software). Calibrations (conversion of R 340/380 values into molar calcium concentrations) were performed using a Fura-2 calcium imaging calibration kit (Molecular Probes, Inc, Eugene OR, USA) as described previously (Grynkiewicz et al., 1985). Basal $[Ca^{2+}]_i$ was determined from the initial 10 images of each cell recording. A $[Ca^{2+}]_i$ signal was defined as an increase in R 340/380 that clearly correlated with the time of application of IFN-γ. The increase in $[Ca^{2+}]_i$ in response to IFN-γ application was calculated as the difference between the basal $[Ca^{2+}]_i$ and the values obtained 10 min after a 3-min treatment with IFN-γ.

2.7. Cell viability

Cell viability was determined by colorimetric measurements of the reduction product of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetra-zolium bromide (MTT). After treatment with IFN-γ, LPS and an antidepressant (paroxetine, sertraline, agomelatine or bupropion), the original medium was removed from the cells in 96-well plates, and the cells were incubated for 2 h at 37 °C in the presence of phenol red free minimum essential medium (Invitrogen Corporation, NY.) containing 0.5 mg/mL MTT. An aliquot (100 μL) of MTT lysis buffer (5% sodium dodecylsulfate (SDS) and 5 mM HCl) was then added to each well, and the plates were incubated at 37 °C overnight to dissolve the formazan that had formed in the wells. MTT is reduced to formazan in the mitochondria of living cells. Reduced MTT was measured by means of a plate reader (Labsystems Multiscan MS, Frankfurt, Germany) at a wavelength of 570 nm.

2.8. Immunocytochemistry

The cultured 6-3 microglial cells and primary microglial cells were maintained on coverslips or on Aclar plastic film. After two rinses in PBS, the cells were fixed with 4% paraformaldehyde for 10 min, and then rinsed with PBS for 10 min. Indirect immunofluorescence was performed using a rabbit anti-serotonin transporter (5-HTT) polyclonal antibody (1:200; Alpha Diagnostic, San Antonio, TX, USA), a mouse anti-OX42 monoclonal antibody (1:100; Serotec, Oxford, UK), or a rat anti-OX42 monoclonal antibody (1:200; Serotec, Oxford, UK). Cells were incubated in these primary antibodies diluted in 0.1% Triton-X 100 in PBS containing 5% normal goat serum at 4 °C overnight. After rinsing twice with PBS for 5 min, fluorescein isothiocyanate (FITC)- or Texas red-conjugated secondary antibodies (Southern Biotech, Birmingham, AL, USA) were used for detection, then cells were examined using a microscope (Olympus BX50).

2.9. Statistics

All data are represented as the means ± Standard Error of Means (S.E.M.) and were analyzed by a one-way analysis of variance (ANOVA). Tukey's honestly significant difference test was used for post hoc comparisons. Significance was established at a level of $p<0.05$. All statistical analyses were performed with SPSS version 13.0 for windows.

3. Results

3.1. The effect of paroxetine and sertraline on NO, TNF-α and IL-4 release by IFN-γ-activated microglia

To determine the effect of antidepressants on IFN-γ-induced NO release in microglial cells, the 6-3 microglial cells were pre-treated with DMSO (0.025%), paroxetine (0.5 or 5 μM), sertraline (0.5 or 5 μM), agomelatine (1 or 10 μM), or bupropion (1 or 10 μM) for 24 h, followed by treatment with IFN-γ (50 U/mL), in the continued presence of each drug, for 24 h. NO release was then assayed by measurement of the accumulation of NO_2. Both paroxetine and sertraline significantly inhibited NO release in a dose-dependent manner when compared with either of the positive controls (treatment with IFN-γ alone or with DMSO + IFN-γ). (Fig. 1) Paroxetine and sertraline had no effect on NO release in the absence of IFN-γ (data not shown). In contrast, neither bupropion nor agomelatine had any inhibitory effect on the release of NO (Fig. 1). The treatment of bupropion or agomelatine alone did not have any effect on NO release in the absence of IFN-γ (data not shown).

To determine the effect of antidepressants on IFN-γ-induced TNF-α release, the 6-3 microglial cells were pre-treated with DMSO (0.025%), paroxetine (0.5 or 5 μM) or sertraline (0.5 or 5 μM) for 24 h, followed by treatment with IFN-γ (50 U/mL) in the continued presence of each drug for 24 h. TNF-α release was then assayed by an ELISA assay. Both paroxetine and sertraline strongly inhibited the release of TNF-α in a dose-dependent manner when compared with either of the positive controls (treatment with IFN-γ alone or with DMSO + IFN-γ) (Fig. 2). Neither paroxetine nor sertraline had any effect on the release of TNF-α in the absence of IFN-γ (data not shown). IFN-γ did not induce IL-4 production from 6-3 microglia (Fig. 3). Neither paroxetine nor sertraline had any effect on the release of IL-4 in the absence of IFN-γ (Fig. 3).

3.2. The effect of paroxetine and sertraline on NO and TNF-α release by LPS-activated rat primary microglia

We next confirmed that the effects of paroxetine and sertraline that were observed in the microglial cell line could be reproduced in rat primary microglial cells. Treatment with both doses of LPS (50 ng/mL

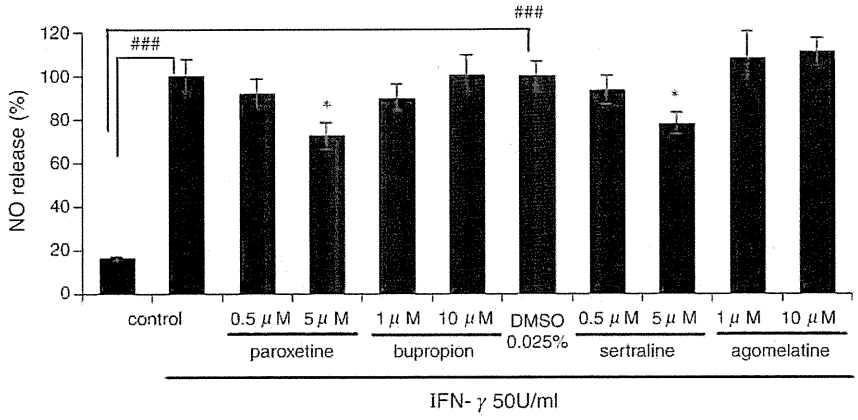

Fig. 1. The effect of antidepressants on NO release by IFN-γ-activated 6-3 murine microglia. The 6-3 microglial cells were pre-treated with paroxetine (0.5 or 5 μM) or bupropion (1 or 10 μM) or sertraline (0.5 or 5 μM) or agomelatine (1 or 10 μM) for 24 h and were then treated with each drug and IFN-γ (50 U/mL) for 24 h. NO release was then determined using the Griess assay. The results are expressed as a percentage of the value of the IFN-γ or IFN-γ + DMSO treatment groups which were assigned a value of 100%. All data are represented as the means (S.E.M.) of three independent experiments (n = 18 for paroxetine, n = 9 for bupropion, n = 21 for sertraline and n = 6 for agomelatine). ###P<0.001 in comparison to the control group. *P<0.05; in comparison to the IFN-γ or IFN-γ + DMSO treatment groups.

and 1 μg/mL) induced NO production (Fig. 4a). LPS treatment had no effect on microglial cell viability. (Fig. 4b) In a similar type of assay to that used for 6-3 microglial cells, paroxetine and sertraline also significantly inhibited NO release by rat primary microglial cells stimulated with LPS (1 μg/mL) (Fig. 4c) In contrast, the SSRIs had no effect on NO production by those stimulated with lower dose of LPS (50 ng/mL) (Fig. 4d). Neither paroxetine nor sertraline inhibited TNF-α release by rat primary microglia (Fig. 5).

3.3. Serotonin transporters in microglial cells

Since paroxetine and sertraline are both typical SSRIs, we wished to determine if the effect of these drugs on microglial cells might be mediated by 5-HTT. We first determined if 5-HTT was expressed in microglial cells, using immunocytochemistry. The result of this experiment suggested that both murine 6-3 microglial cells and rat primary microglial cells do express serotonin transporters. (Fig. 6a and b respectively).

We next investigated if the inhibitory effects of the SSRIs on NO release by microglia are mediated by 5-HTT. The 6-3 microglial cells were therefore pre-treated with DMSO (0.025%), paroxetine (5 μM) or sertraline (5 μM) in the presence or absence of 1 mM of 5-HT. After 24 h incubation, the cells were treated with IFN-γ (50 U/mL) in the continued presence of the drugs for a further 24 h. The media were then collected and assayed for NO accumulation as described above. This experiment showed that the inhibitory effects of paroxetine or sertraline on NO release by IFN-γ-activated microglia were not reversed even by a large (200X) excess of 5-HT, which may saturate 5-HTT in microglia. (paroxetine vs 5-HT + paroxetine; $p = 0.175$) (sertraline vs 5-HT + sertraline; $p = 0.972$), (Fig. 7). This result possibly suggests that the inhibitory effects of paroxetine and sertraline on microglia may not be mediated by 5-HTT.

3.4. The intracellular signaling mechanism by which IFN-γ induces NO and TNF-α release from activated microglia

The significant induction of NO and TNF-α release from 6-3 murine microglial cells that we observed following IFN-γ

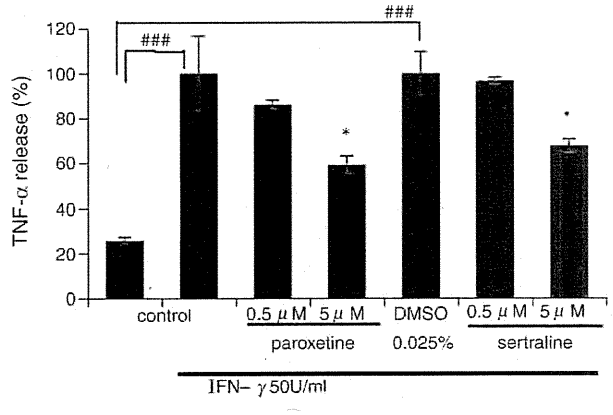

Fig. 2. The effect of antidepressants on TNF-α release by IFN-gamma-activated 6-3 murine microglia. The 6-3 microglial cells were pre-treated with paroxetine (0.5 or 5 μM) or sertraline (0.5 or 5 μM) for 24 h and were then treated with each drug and IFN-γ (50 U/mL) for 24 h. TNF-α release was then determined using an ELISA assay. The results are expressed as a percentage of the values of the IFN-γ or IFN-γ + DMSO treatment groups that were assigned a value of 100%. All data are represented as the means (S.E.M.) of three independent experiments (n = 3 for both paroxetine and sertraline). ###P<0.001 in comparison to the control treatment group. *P<0.05 in comparison to the IFN-γ or IFN-γ + DMSO treatment groups.

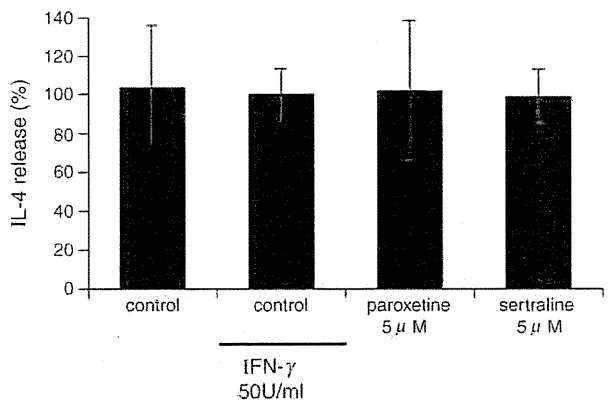

Fig. 3. The effect of IFN-gamma and antidepressants on IL-4 release by 6-3 murine microglia. The 6-3 cells were plated on 96-well tissue culture plates at a density of 1×10^5 per 200 μL per well. The cells were treated in the presence or absence of paroxetine (5 μM), sertraline (5 μM) or IFN-γ (50 U/mL) for 24 h. IL-4 release was then determined using an ELISA assay. The results are expressed as a percentage of the values of control group that were assigned a value of 100%. All data are represented as the means (S.E.M.) of three independent experiments (n = 6 for both paroxetine and sertraline).

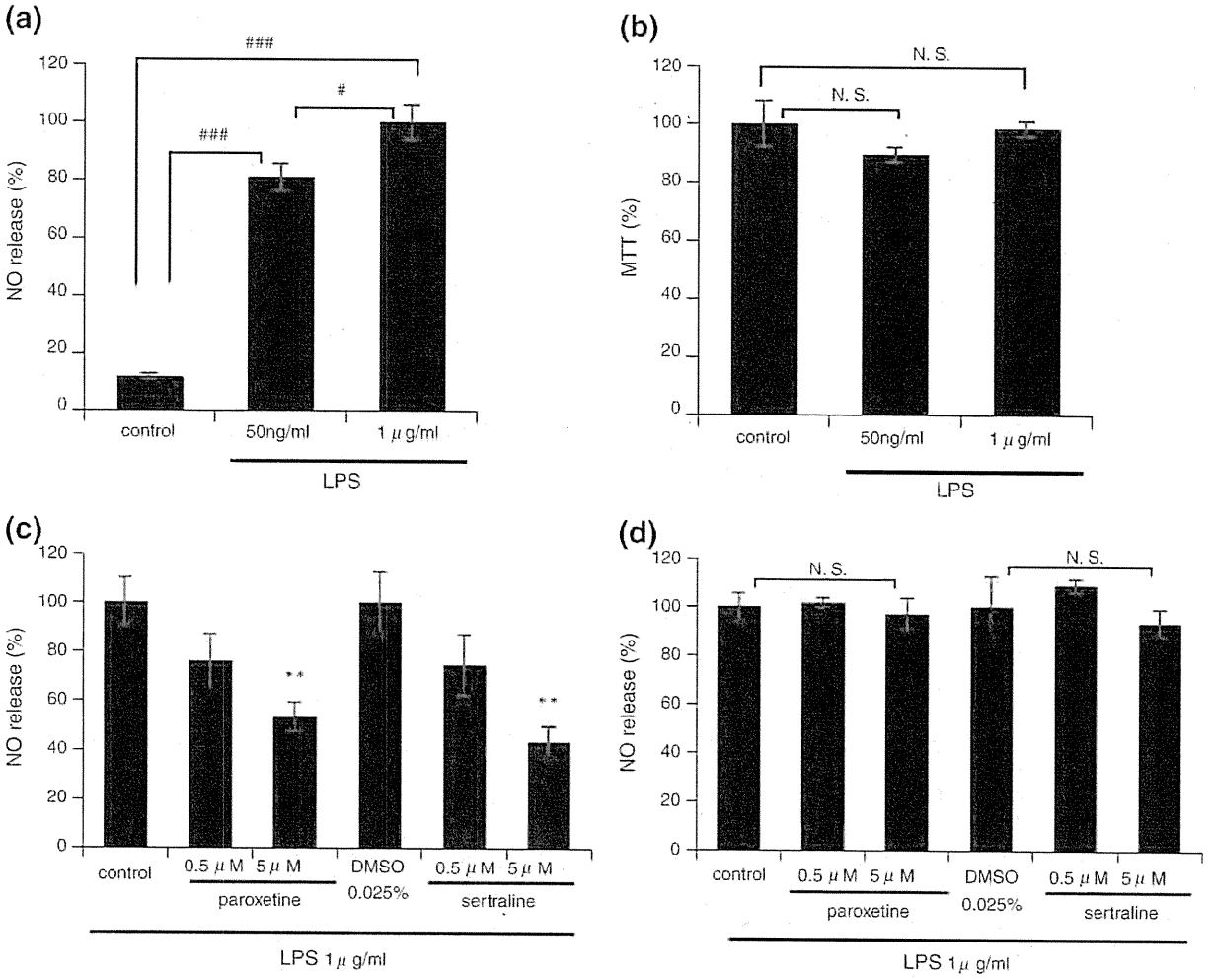

Fig. 4. The effect of antidepressants on NO release by LPS-activated rat primary microglia, and the effect of LPS on microglial cell viability. Rat primary microglial cells pre-treated with paroxetine (0.5 or 5 μM) or DMSO (0.025%) or sertraline (0.5 or 5 μM) for 24 h and were then treated with each drug and LPS (1 μg/mL or 50 ng/mL) for 24 h. NO release was then determined using the Griess assay (a, c and d). The results are expressed as a percentage of the value of the LPS or LPS + DMSO treatment group that was assigned a value of 100%. Cell viability was determined using the MTT assay (b). The results are expressed as a percentage of the value of control group that was assigned a value of 100%. All data are represented as the means (S.E.M.) of three independent experiments (n = 12 for paroxetine and n = 6 for sertraline). ###P<0.001; #P<0.05 in comparison between respective groups. *P<0.05; **P<0.01 in comparison to the LPS or LPS + DMSO treatment groups.

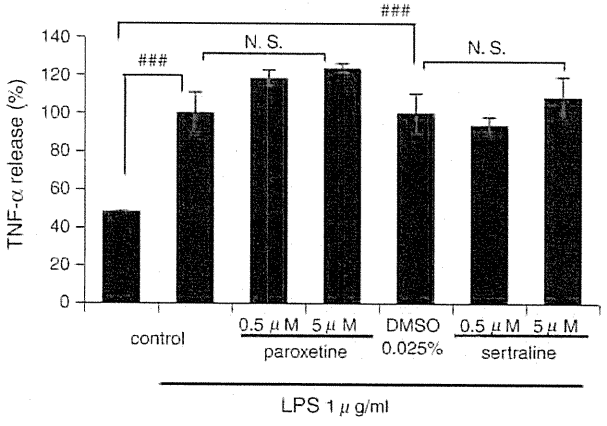

Fig. 5. The effect of antidepressants on TNF-α release by LPS-activated rat primary microglia. Rat primary microglial cells pre-treated with paroxetine (0.5 or 5 μM) or DMSO (0.025%) or sertraline (0.5 or 5 μM) for 24 h and were then treated with each drug and LPS (1 μg/mL) for 24 h. NO release was then determined using the Griess assay. The results are expressed as a percentage of the value of the LPS or LPS + DMSO treatment group that was assigned a value of 100%. All data are represented as the means (S.E.M.) of three independent experiments (n = 3 for paroxetine and sertraline). ###P<0.001 in comparison to the control treatment group.

treatment is consistent with previous reports (Bian et al., 2008; Kato et al., 2007). To determine if the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway, which is known to be activated by IFN-γ might play a role, we pre-treated the 6-3 microglial cells with 0.01 μM and 0.1 μM of a specific inhibitor of the (JAK-STAT) pathway, the JAK inhibitor-1. After 24 hr incubation the cells were treated with IFN-γ (50 U/mL) for a further 24 h in the continued presence of the inhibitor. The media were then collected and the effect of the JAK inhibitor-1 on NO and TNF-α accumulation was assayed as described above. The JAK inhibitor-1 significantly inhibited NO and TNF-α release from IFN-γ-activated microglia (Fig. 8a and b respectively) but did not have any effect on cell viability (data not shown). The treatment of JAK inhibitor-1 alone did not have any effect on NO and TNF-α release in the absence of IFN-γ (data not shown). These results suggest that the JAK-STAT pathway plays a key role in IFN-γ-induced activation of murine microglial cells. These results further suggest that the inhibitory effects of paroxetine and sertraline on NO and TNF-α release by IFN-γ-activated microglia may possibly occur by inhibition of the JAK-STAT pathway.

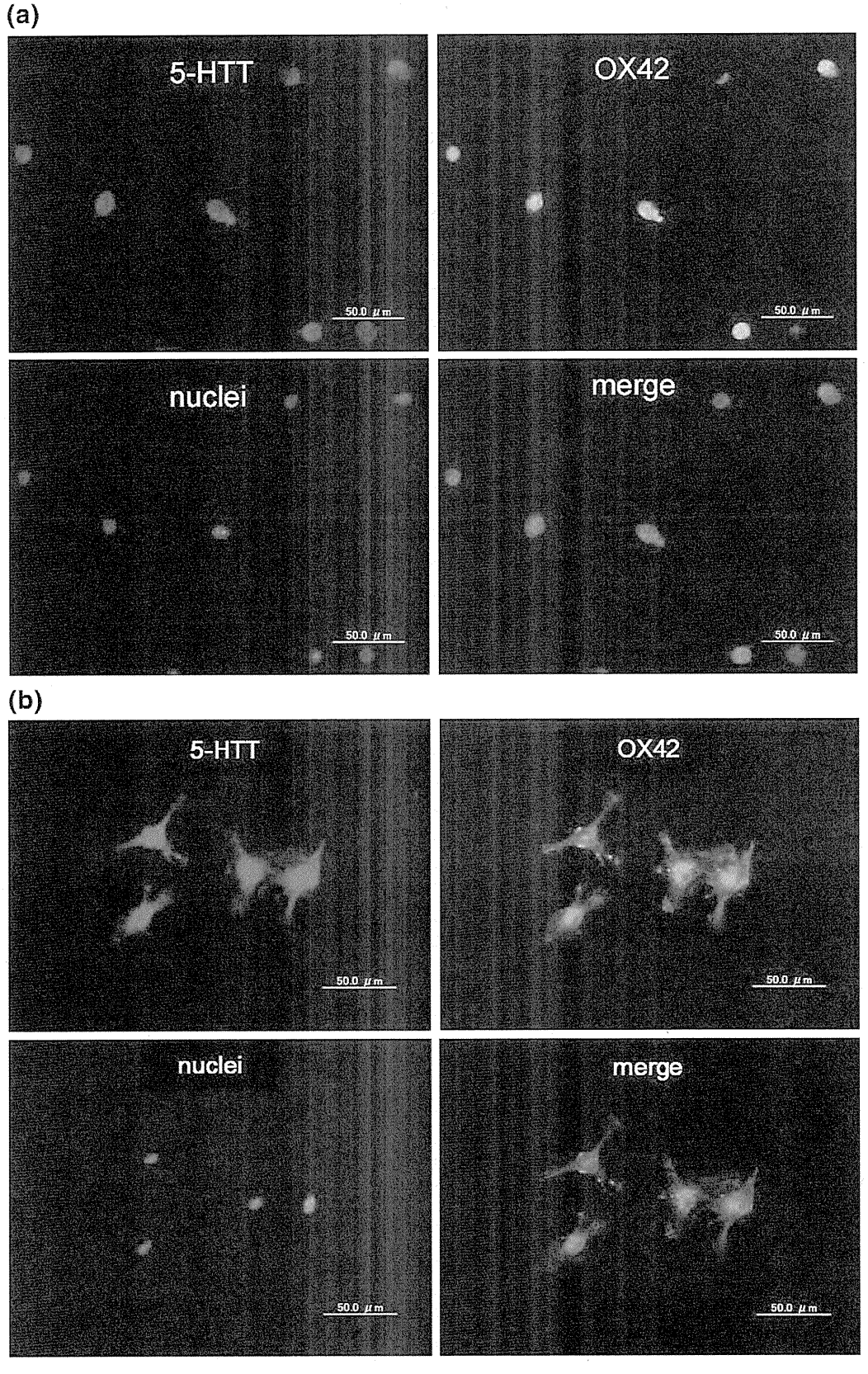

Fig. 6. The expression of serotonin transporters in murine 6-3 microglial and rat primary microglial cells. The expression of serotonin transporters was assayed in (a) murine 6-3 microglial cells and (b) rat primary microglial cells by double-immunolabeling with a polyclonal rabbit anti-serotonin transporter antibody and a monoclonal mouse OX42 antibody as described in Methods. Nuclei were stained by DAPI. The cells were then examined by microscopy and merged images are shown. Scale bar, 50 μm.

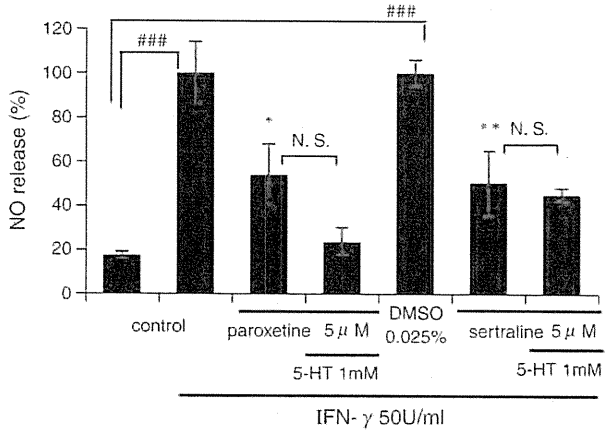

Fig. 7. The effect of paroxetine and sertraline on NO release by 6-3 murine microglia in the presence or absence of a large excess of 5-HT. The 6-3 microglial cells were pre-treated with paroxetine (5 μM) or sertraline (5 μM) in the presence or absence of serotonin (5-HT, 1 mM) for 24 h and were then treated with each drug and IFN-γ (50 U/mL) for 24 h. NO release was then determined using the Griess assay and the results are expressed as a percentage of the values of the IFN-γ (paroxetine) or IFN-γ + DMSO (sertraline) treatment groups that were assigned a value of 100%. All data are represented as the means (S.E.M.) of three independent experiments (n = 18 for paroxetine and n = 9 for sertraline). ###P<0.001 in comparison to the control treatment group. *P<0.05; **P<0.01; in comparison to the IFN-γ (paroxetine) or IFN-γ + DMSO (sertraline) treatment groups.

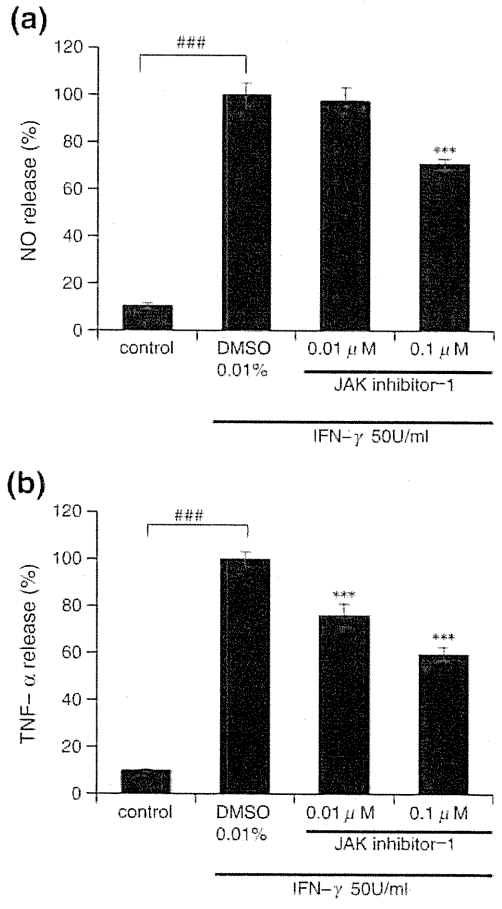

Fig. 8. The effect of the JAK inhibitor-1 on NO and TNF-α release by IFN-γ-activated microglia. NO (a) and TNF-α (b) release were determined using the Griess assay and an ELISA, respectively. The 6-3 microglial cells were pre-treated with the JAK inhibitor-1 (0.01 or 0.1 μM) for 24 h, and were then treated with each drug and IFN-γ (50 U/mL) for 24 h. The results are expressed as a percentage of the value of the IFN-γ + DMSO treatment group that was assigned a value of 100%. All data are represented as the means (S.E.M.) of three independent experiments (n = 6). ###P<0.001 in comparison to the DMSO control group. ***P<0.001 in comparison to the IFN-γ + DMSO treatment group.

3.5. A 24 hr treatment with paroxetine or sertraline attenuates the mobilization of intracellular Ca^{2+} induced by IFN-γ application in murine microglial cells

In human microglia, IFN-γ rapidly induces a progressive increase in $[Ca^{2+}]_i$ and IFN-γ acts solely through influx of Ca^{2+} (Franciosi et al., 2002). The $[Ca^{2+}]_i$ is also very important for the regulation of microglial activation including regulation of the release of NO and cytokines (Hoffmann et al., 2003).

To determine if the antidepressant drugs might influence the mobilization of intracellular Ca^{2+} induced by IFN-γ treatment of murine 6-3 microglial cells, we measured the effect of a 24 hr treatment with paroxetine (5 μM), sertraline (5 μM), agomelatine (10 μM) or bupropion (10 μM) on this phenomenon. As shown in Fig. 8a, IFN-γ (50 U/mL) rapidly increased $[Ca^{2+}]_i$ in the 6-3 cells (n = 113 cells) and, once $[Ca^{2+}]_i$ rose, it gradually increased without attenuation. The increase in $[Ca^{2+}]_i$ was sustained for >40 min after washout of IFN-γ. Thus, IFN-γ induces a sustained intracellular Ca^{2+} elevation in murine microglia as has been previously shown in human microglia (Franciosi et al., 2002).

Pretreatment of the microglial cells with each antidepressant (paroxetine, sertraline, agomelatine or bupropion) for 24 h did not inhibit IFN-γ (50 U/mL)-induced sustained intracellular Ca^{2+} elevation (n = 15 for paroxetine, 22 for sertraline, 37 for agomelatine, and 33 for bupropion.). However, the amplitude of the $[Ca^{2+}]_i$ increase induced by IFN-γ was very different for each inhibitor group. Treatment with agomelatine or bupropion did not affect the amplitude of the increase in $[Ca^{2+}]_i$ induced by IFN-γ in murine microglia (101.1 ± 7.84 nM and 93.81 ± 6.55 nM following control or bupropion treatment respectively; p = 0.24 and 120.9 ± 8.02 nM and 110.10 ± 17.72 nM following DMSO or agomelatine treatment respectively; p = 0.29) (Fig. 9b). In contrast, pretreatment with paroxetine or sertraline (5 μM for 24 h) significantly reduced the amplitude of the increase in $[Ca^{2+}]_i$ induced by IFN-γ (101.1 ± 7.84 nM and 43.69 ± 3.88 nM following treatment with control or paroxetine respectively; p<0.001), (120.9 ± 8.02 nM and 40.44 ± 10.77 nM following treatment with DMSO and sertraline respectively; p<0.001) (Fig. 9b).

These results suggest that 24-hr pretreatment with paroxetine (5 μM) or sertraline (5 μM) attenuates the mobilization of intracellular Ca^{2+} induced by IFN-γ application in murine microglial cells while 24-hr pretreatment with bupropion (10 μM) or agomelatine (10 μM) does not.

3.6. Effect of the antidepressants on cell viability

The effect of the antidepressants on the cell viability of 6-3 microglial cells was assayed by pretreatment of the cells with DMSO (0.025%), paroxetine (0.5 or 5 μM), agomelatine (1 or 10 μM), sertraline (0.5 or 5 μM), or agomelatine (1 or 10 μM) for 24 h, following which the cells were treated with IFN-γ (50 U/mL) for 24 h in the continued presence of the drugs. IFN-γ treatment alone did not have any significant effect on cell viability and pretreatment with none of the antidepressants showed no significant cytotoxicity towards activated microglia (Fig. 10) (control vs IFN-γ; p = 0.144) (IFN-γ + paroxetine 5 μM vs IFN-γ; p = 0.984) (IFN-γ + bupropion 10 μM vs IFN-γ; p = 0.994) (sertraline 5 μM + IFN-γ vs DMSO + IFN-γ; p = 0.522) (agomelatine 10 μM + IFN-γ vs DMSO + IFN-γ; p = 1.000).

4. Discussion

In the present study, paroxetine and sertraline significantly inhibited the generation of NO and TNF-α from IFN-γ-activated microglia as well as to suppress the IFN-γ-induced elevation of $[Ca^{2+}]_i$ in microglia. Neither paroxetine nor sertraline were toxic to microglial cells at their effective concentrations. In addition,

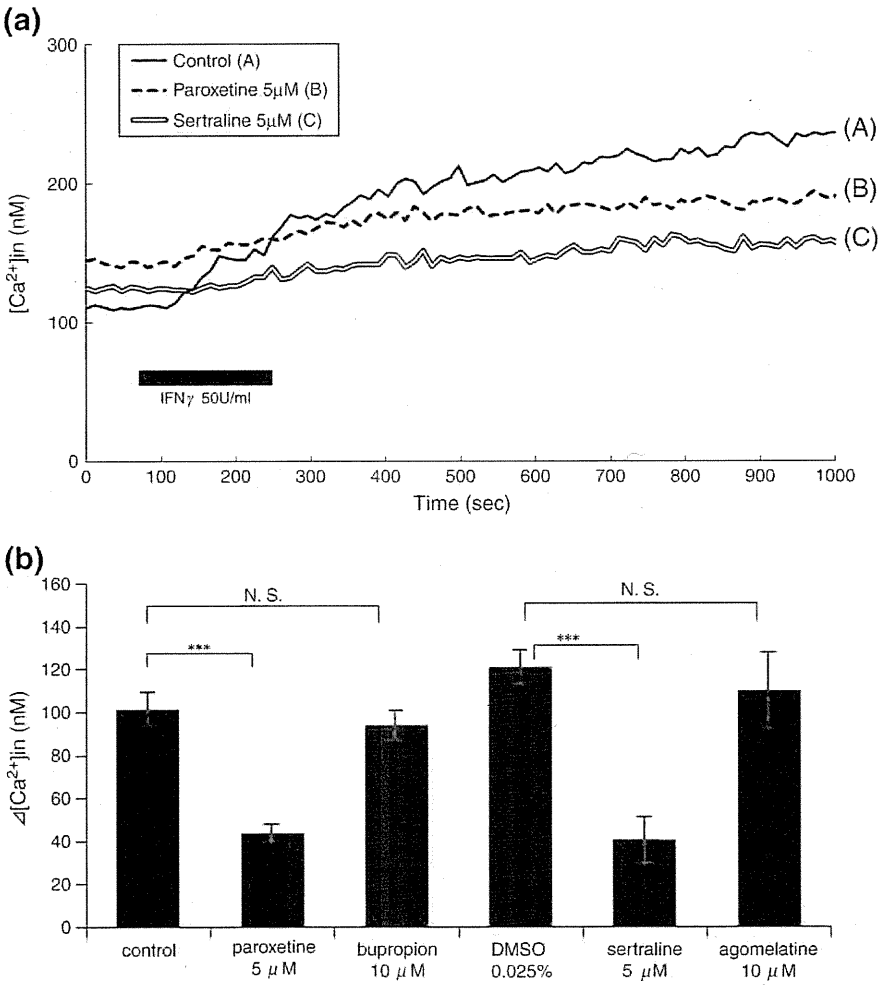

Fig. 9. The mobilization of intracellular Ca2+ induced by IFN-gamma-activated microglia. a) Sustained elevation of intracellular Ca2+ induced by IFN-γ application in the murine 6-3 microglial cells. Microglial cells were activated by IFN-γ (50 U/mL) for 3 min. The data shows the average trace of [Ca2+]I of control group (A). Microglial cells were pre-treated with paroxetine (5 μM) or with sertraline (5 μM) for 24 h followed by activation by IFN-γ (50 U/mL) for 3 min. The data shows the average trace of [Ca2+]I of paroxetine pre-treated group (B), and 5 μM sertraline pre-treated group (C). b) Microglial cells were pre-treated with paroxetine (5 μM) or bupropion (10 μM), or with sertraline (5 μM), agomelatine (10 μM) or DMSO (0.025%) for 24 h followed by activation by IFN-γ (50 U/mL) for 3 min. The increase in the [Ca2+]i in response to IFN-γ application is calculated as the difference between the basal [Ca2+]i and the value of [Ca2+]i measured 10 min after a 3-min treatment with IFN-γ. Data are represented as the means (S.E.M.) for each experiment (n = 15 for paroxetine, n = 22 for sertraline, n = 37 for agomelatine, and n = 33 for bupropion). ***P<0.001 in comparison to the IFN-γ treatment group or the DMSO plus IFN-γ treatment group.

these antidepressants significantly inhibited NO release from rat primary microglia activated by LPS (1 μg/mL), while they had no effect on the NO release from those activated by lower dose of LPS (50 ng/mL), and the mechanism underlying this difference remains to be elucidated. Although neither paroxetine nor sertraline completely normalized activation levels of IFN-γ-stimulated microglia, their inhibitory effects on microglial activation that has been demonstrated in our study may have important implications for the therapeutic application of these antidepressants in a variety of disorders involving depression.

Therapeutic doses of paroxetine, sertraline and bupropion reach levels of 0.19–0.32 μM, 0.029–0.14 μM, <0.36 μM respectively in serum (Baumann et al., 2004). Psychotropic drugs are reported to accumulate in brain tissue at levels that are 10- to 40-fold higher in brain tissue than in serum (Baumann et al., 2004). In the present study, the final concentration of paroxetine and sertraline was 0.5–5 μM and the final concentration of bupropion and agomelatine was 1–10 μM. Therefore, those may be similar to the putative level of the antidepressants within brain tissue following therapeutic administration.

4.1. Effects of antidepressants on the regulation of $[Ca^{2+}]_i$ and the intracellular signaling mechanism in IFN-γ-activated microglia

IFN-γ has been reported to induce Ca^{2+} influx in human microglia (Franciosi et al., 2002). We also observed that IFN-γ induces sustained $[Ca^{2+}]_i$ elevation in murine microglia. It has been demonstrated that treatment with the calcium chelator 1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA) can inhibit the release of NO and cytokines from LPS-activated microglia whereas ionomycin, an ionophore that elevate Ca^{2+}, has no effect on the release of NO or cytokines from LPS-activated microglia. These data indicate that increased $[Ca^{2+}]_i$ is required, but by itself is not sufficient, for the release of NO and certain cytokines from activated microglia (Hoffmann et al., 2003). We observed that pretreatment with paroxetine or sertraline attenuated the mobilization of intracellular Ca^{2+} induced by IFN-γ. In contrast, pretreatment with either agomelatine or bupropion had no effect on NO production from activated microglia, or on the elevation of intracellular $[Ca^{2+}]_i$ induced by IFN-γ.

We have previously reported that generation of NO and TNF-α from IFN-γ-activated microglia was significantly inhibited by specific

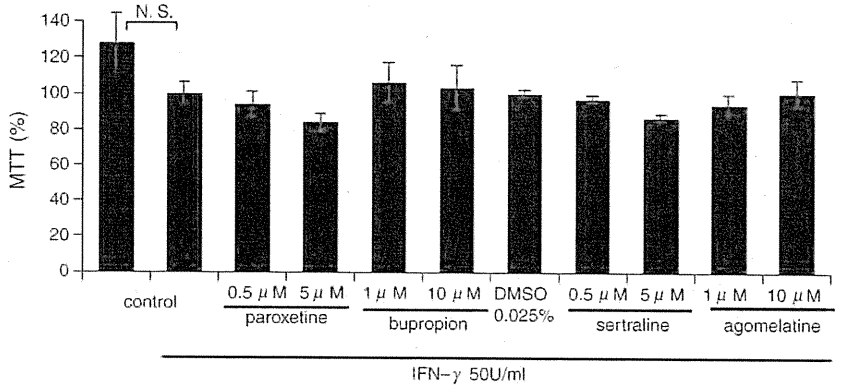

Fig. 10. The effect of antidepressants on cell viability. Cell viability was determined by colorimetric measurement of the reduction product of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetra-zolium bromide (MTT). The 6-3 microglial cells pre-treated with paroxetine (0.5 or 5 μM) or bupropion (1 or10 μM),or DMSO (0.025%) or sertraline (0.5 or 5 μM) or agomelatine (1 or 10 μM), for 24 h and were then treated with IFN-γ (50 U/mL) for 24 h. The results are expressed as a percentage of the IFN-γ treatment group (paroxetine and bupropion) or the DMSO plus IFN-γ treatment group (sertraline and agomelatine) that were each assigned a value of 100%. All data are represented as the means (S.E.M.) of three independent experiments (n = 18 for paroxetine, n = 9 for bupropion, n = 21 for sertraline, and n = 6 for agomelatine). ###P<0.001 in comparison to the control group.

inhibitors of PKC, p38 MAPK and ERK 1/2, all of which appear to be activated by the elevated $[Ca^{2+}]_i$ (Kato et al., 2008). In the present study, JAK inhibitor-1 also significantly inhibited production of NO and TNF-α from IFN-γ-activated microglia. The biological effects of IFN-γ are elicited through the activation of intracellular signaling pathways, including JAK-STAT pathway (Gough et al., 2008). In this pathway, the phosphorylated STAT1 homodimer translocates to the nucleus where it initiates gene transcription, and the influx of Ca^{2+} induced by IFN-γ is required for the ser-727 phosphorylation of STAT1 in NIH 3 T3 cells (Nair et al., 2002). Based on these results, we speculate that paroxetine and sertraline may inhibit signaling pathways including PKC, p38 MAPK, ERK 1/2, and JAK-STAT pathways in microglia by inhibition of IFN-γ-induced elevation of $[Ca^{2+}]_i$ that ultimately results in reduced production of NO and TNF-α.

4.2. The role of 5-HTT in microglial activation

We considered 5-HTT to be the molecular targets for inhibitory effects of SSRIs on microglial activation. We therefore demonstrated the existence of 5-HTT in microglial cells by immunocytochemistry. Peritoneal macrophages are reported to express 5-HTT (Rudd et al., 2005). However, to our knowledge, this is the first report of the existence of 5-HTT in microglial cells. Our data possibly suggest that the inhibitory effects of paroxetine and sertraline on microglial activation may not be mediated by 5-HTT since their effects were not reversed even by a 200-fold excess of 5-HT, which may saturate 5-HTT in microglia. Such 5-HTT-independent effects of SSRIs have been reported in Burkitt lymphoma cells (Serafeim et al., 2003). However, the molecular targets for the observed effects of paroxetine and sertraline on microglia are still uncertain. Further study is thus required to determine those.

4.3. Anti-inflammatory effects of antidepressants, which may be beneficial in the treatment of depression and a variety of disorders

A general relationship between depression and neurogenesis has been described (Warner-Schmidt and Duman, 2006) while some behavioral effects of certain antidepressants appear to be neurogenesis-independent (Holick et al., 2008). Fluoxetine has been reported to target early progenitor cells in the adult brain (Encinas et al., 2006). However, antidepressants may also indirectly stimulate neurogenesis through inhibition of activated microglia-derived release of NO and inflammatory cytokines such as TNF-α (Iosif et al., 2006), both of which have inhibitory effects on neurogenesis (Kaneko et al., 2006; Monje et al., 2003; Ekdahl et al., 2003; Cardenas et al., 2005; Vallieres et al., 2002). Therefore, the effect of the antidepressants assayed in this study on the release of NO and TNF-α suggests the possibility that these antidepressants may also indirectly stimulate neurogenesis.

Recent diffuse tensor imaging studies have shown white matter abnormalities of frontal or temporal brain regions in elderly depressed patients as well as in young, treatment-naïve depressed patients (Ma et al., 2007, Nobuhara et al., 2006, Taylor et al., 2004). Microglial activation in the CNS has been implicated in the pathogenesis of white matter disorders and it has recently been reported that microglial cytotoxicity of oligodendrocytes is mediated through free radical-related molecules such as NO and peroxynitrite generated by activated microglia (Merrill et al., 1993, Li et al., 2005) and by inflammatory cytokines such as TNF-α and IFN-γ (Buntinx et al., 2004b, Buntinx et al., 2004a). Our data, that show an effect of parexetine and sertraline on NO and TNF-α produced by microglia may, therefore, suggest that these antidepressants may ameliorate white matter disorders that are observed in depression.

Depression, particularly in elderly patients, can manifest itself as a dementia-like syndrome, which is referred to as depressive pseudodementia. Furthermore, a history of depression is reported to be a risk factor for AD. Neuoinflammation may thus be a common key pathology of depression and dementia (Leonard 2007). Interestingly, pre-symptomatic and chronic treatment with paroxetine has been shown to decrease AD-like pathology and to reverse memory impairment in 3× transgenic AD mice (Nelson et al., 2007). Furthermore, antidepressant treatment has recently been reported to be effective against the cognitive decline of both patients with mild cognitive impairment and patients with AD (Mossello et al., 2008, Mowla et al., 2007). Continued long-term antidepressant treatment has been reported to be associated with a reduced rate of dementia (Kessing et al., 2009). The observed effect of the antidepressants assayed in this study on the production of inflammatory molecules may therefore suggest a potential use for these antidepressants in the prevention or treatment of dementia.

Antidepressants are thought to affect mainly neurons or neural networks over a long period of time. However, the present study has demonstrated that paroxetine and sertraline have a strong anti-inflammatory effect via the inhibition of microglial activation, which may have a deleterious effect on the brains of patients with depression. The mechanisms by which these antidepressants inhibit microglial activation appear to be similar to that of aripiprazole (Kato et al., 2008). Interestingly, aripiprazole is approved by the U.S. Food and Drug Administration (FDA) as the first antipsychotic to be used as an adjunct to antidepressant medications in the treatment of unresponsive major depression (Mohamed et al., 2009, Weber et al.,

2008). In the present study, bupropion and agomelatine did not inhibit NO production by IFN-γ activated microglia while paroxetine and sertraline did. A similar lack of inhibition of the production of NO and TNF-α by atomoxetine or desipramine has been reported (O'Sullivan et al., 2009). In the present study, microglial cells were pre-treated with antidepressants for 24 h, so this inhibitory effect of SSRIs on microglial activation could be expressed as an acute effect. However, it is well known that antidepressants take a few weeks to exert a clinical effect (Thompson 2002). Based on these results, we speculate that inhibition of microglial activation may be an early step in the antidepressant mechanism, although not in itself sufficient to produce an antidepressant effect.

In the present study, we activated microglial cells with IFN-γ, which is a typical Th1 cytokine, and some reports have suggested a relationship between depression and IFN-γ. Suicide in depression has been reported to be related to a central nervous serotonin deficiency, possibly induced by IFN-γ mediated IDO activation (Schwarz et al., 2001a; Schwarz et al., 2001b). IFN-γ itself has also been reported to play a role in modulating fear and anxiety (Hurlock 2001). We thus used IFN-γ as an activator of the microglia. Whereas IFN-γ is almost absent in normal brain tissue, it is reported that expression of IFN-γ increases after experimental brain ischemia, and brain-invading T cells are major source of IFN-γ in the ischemic brain (Liesz et al., 2009). Brain-invading T cells and IFN-γ play important role also in the pathology of multiple scrlosis (MS) (Hauser and Oksenberg 2006). It is reported that prevalence of depression of patients with MS or stroke is higher when compared with normal controls (Mohr et al., 2006, Robinson 2003). Depression in MS responds well to antidepressants while it is unlikely to improve without effective treatment of MS (Mohr et al., 2006). SSRIs are also reported to be effective to treat poststroke depression (Gabaldon et al., 2007). Therefore, inhibitory effect of SSRIs on microglial activation induced by IFN-γ may possibly be beneficial especially for depressive symptom of patients with stroke or MS. On the other hand, it is reported that CNS-infiltrating peripheral macrophages also contribute to CNS inflammation, and may also contribute to axonal damage in the pathology of MS (Hendriks et al., 2005, Schmid et al., 2009). Further study is thus required to investigate effects of antidepressants on function of other immune cells, such as macrophages or T cells.

Another study has suggested that TNF-α can induce depression-like symptoms in mice (Simen et al., 2006). Our results showed that paroxetine and sertraline inhibited TNF-α production by IFN-γ-activated microglia. Recent studies have reported that TNF-α antagonists are effective for depression associated with psoriasis and rheumatoid arthritis (Tyring et al., 2006, Uguz et al., 2009). Immunosuppressive agents themselves may thus be useful for the treatment of depression.

In the present study IFN-γ did not induce the release of anti-inflammatory cytokine IL-4 from microglia. Furthermore, other studies have suggested that the production of neuroprotective factors by microglia was found to be reduced following IFN-γ stimulation (Graeber and Streit, 2010; Moran et al., 2007). We thus focused on inflammatory form of microglial activation. However, activation of microglia, as well as that of macrophage, is not always neurotoxic but sometimes neuroprotective through the production of neurotrophins (Hendriks et al., 2005; Schwartz et al., 2006). Therefore, we should further investigate effects of SSRIs on neuroprotective form of microglial activation, and on homeostatic functions of microglia in non-inflamed CNS.

5. Conclusion

Inhibitory effects of paroxetine and sertraline on microglial activation reported in this paper shed some new light on the design of therapeutic strategies for the treatment of depression. Future study should focus on a detailed clarification of the molecular mechanism(s) by which antidepressants inhibit microglial activation and on confirmation of the results reported herein in *in vivo* studies.

Conflict of interest

The authors have no conflict of interest.

Acknowledgments

The present study was supported partly by a grant-in-aid from the Japan Society for the Promotion in Science. The authors thank Prof. Makoto Sawada of Nagoya University for providing us with the microglial cell line 6-3.

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PSYCHOGERIATRICS
The Official Journal of the Japanese Psychogeriatric Society

doi:10.1111/j.1479-8301.2011.00358.x PSYCHOGERIATRICS 2011; 11: 68–76

REVIEW ARTICLE

Post-stroke depression and apathy: Interactions between functional recovery, lesion location, and emotional response

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Received 2 February 2011; accepted 16 February 2011.

Key words: *apathy, depression, insistence on recovery, rehabilitation, stroke.*

Abstract

Depression and apathy are often observed after stroke and are often confused with one another. In the present review, we argue that the current concept of 'post-stroke depression' (PSD) in fact consists of two core symptoms or syndromes: (i) affective (depressive) PSD; and (ii) apathetic PSD. We argue that these two core symptoms are each associated with a different underlying neuroanatomical mechanism, a pattern that influences functional recovery. Post-stroke disabilities can provoke several distinct emotional responses, some of which are associated with severe depression. We examined one of these emotional responses previously, namely 'insistence on recovery', which was believed to be a negative indicator of functional improvement in disabled stroke patients. However, an appropriate level of insistence on recovery may, in fact, be associated with reduced depression and apathy, resulting in enhanced recovery from stroke-related disabilities. Improvements in physical disabilities (trunk stability or activities of daily living, such as walking) also reduce depression and apathy. Therefore, the experience of PSD/apathy may be intertwined with various initial emotional responses and improvements in physical functioning. Effective treatment of PSD/apathy requires a multidisciplinary approach, such that neuroanatomical/neurobiological, emotional, and physical (rehabilitation) domains are all addressed.

INTRODUCTION

Depression is a common neuropsychiatric consequence of stroke, one that has been reported to negatively affect functional and cognitive recovery.[1–3] Apathy also occurs frequently after stroke and is defined as reduced motivation to engage in activities or a general lack of initiative.[4–6] A post-stroke apathetic state can prevent patients from engaging in rehabilitation programs, resulting in delayed physical and psychosocial recovery.

The symptom of apathy is thought to partially overlap with the experience of depression, a view particularly prevalent in the psychiatric field. However, several recent neurological/neuroanatomical studies have revealed important differences between the two syndromes. The terms 'post-stroke depression' (PSD) and 'apathy' may be routinely confused with one another, or used interchangeably despite differences between the two phenomena.[7] Herein, we attempt to clarify these differences.

Residual physical disabilities result in distress and depression for many stroke patients. Mourning the loss of one's previous health status is thought to be typical and normative,[8,9] although few studies have examined correlations between indicators of the mourning process and depression/apathy. We also discuss the importance of care for stroke patients from the viewpoint of emotional responses, particularly a variable dubbed 'insistence on recovery', which refers to stroke patients' insistence that they will recover fully even in the face of serious stroke-related consequences.

DEFINITION OF PSD

In the 1980s, several clinical studies using computed tomography (CT) scanning found that depressive symptoms occur quite frequently after stroke. The concept of PSD has since received some clinical and research attention.[7,10,11] Robinson *et al*. reported a significant relationship between strokes affecting the frontal lobe of the left hemisphere and PSD, postulating a 'left frontal lobe theory'.[10,11] This theory is now considered to be widely supported. In the 1990s, magnetic resonance imaging (MRI) research supported a relationship between geriatric (late life) depression and deep white matter hyperintensity, and thus the concept of 'vascular depression' appeared.[1,12] According to this viewpoint, this particular type of depression is the consequence of brain damage induced by symptomatic or asymptomatic ischemic events. The association between the location of the post-stroke lesion and depressive symptoms has recently started to garner attention.

The clinical symptoms of vascular depression are thought to closely resemble those of major depression. Some previous papers have reported certain typical characteristics of vascular depression. For example, loss of interest and cognitive disturbance are said to be more frequent and more intractable to treatment than is the case in major depression.[1,12–16] In general, loss of interest has proven difficult to treat medically, and thus recovery from vascular depression may be more difficult compared with recovery from major depression.[7]

DEFINITION OF APATHY

Apathy is often observed after stroke and is defined as reduced motivation to engage in activities or lack of initiative.[4–6] Apathy was commonly thought to be associated with prefrontal lesions or lesions of the basal ganglia, with the symptom profile of conditions such as Parkinson's disease being evoked to support this notion.[4–6,17,18] The diagnostic term 'apathy' was typically applied in the neurological field, rather than in psychological or psychiatric fields (the diagnostic category 'apathy' cannot be found in DSM-IV).[19] However, some symptoms of 'apathy' appear as part of the diagnostic criteria for a major depressive episode in DSM-IV, including markedly diminished interest or pleasure in all, or almost all, activities, fatigue or loss of energy, diminished ability to think or concentrate, and indecisiveness.[19] In the psychological/psychiatric fields, apathy is typically viewed as an aspect of depression.

FREQUENCY OF PSD AND APATHY

Depression and apathy are common neuropsychiatric consequences of stroke during the acute and subacute phases of recovery.[2,3] However, as noted above, there has been some confusion regarding how these terms are used. We attempted to independently examine the occurrence of PSD and apathy during acute or subacute phases of hospitalization after stroke.[20] We assessed depression using the Zung Self-Rating Depression Scale (SDS),[21] and apathy using the Japanese version of the Apathy Scale (AS).[4–6] Results from a sample of 243 post-stroke patients on a rehabilitation unit showed that there were 29 cases (11.9%) of depression without apathy, 48 cases (19.8%) of apathy without depression, and 50 cases (20.6%) in which depression and apathy occurred simultaneously; altogether, depression and apathy were found in 126 patients (51.9%) after stroke.[7,20] In another institution (Hibino Hospital), depression was examined using the Hospital Anxiety and Depression Scale (HADS) and apathy was assessed using the AS. In a sample of 101 stroke patients, very similar results were obtained: there were 12 cases (11.9%) of depression without apathy, 28 cases (27.7%) of apathy without depression, and 15 cases (14.9%) of both depression and apathy.[7] In this series, depression and apathy occurred in a total of 55 patients (54.5%; Fig. 1).[7] Depression and apathy do appear to partially overlap and can co-occur, but they can also occur independently after stroke. Therefore, these symptom patterns appear to constitute relatively independent entities.

DEPRESSION OR APATHY AND FUNCTIONAL RECOVERY AFTER STROKE

Depression and/or apathy are widely thought to have a negative impact on functional recovery after stroke,[2,3] although few studies have examined this possibility directly. We examined the influence of depression and apathy on functional recovery after stroke in 237 Japanese patients.[22] We assessed psychological status using self-rating scales (the SDS for depression and the AS for apathy) and physical disability using the Functional Independence Measurement (FIM), which is an observer-rated multi-item summed rating scale used to evaluate disability in

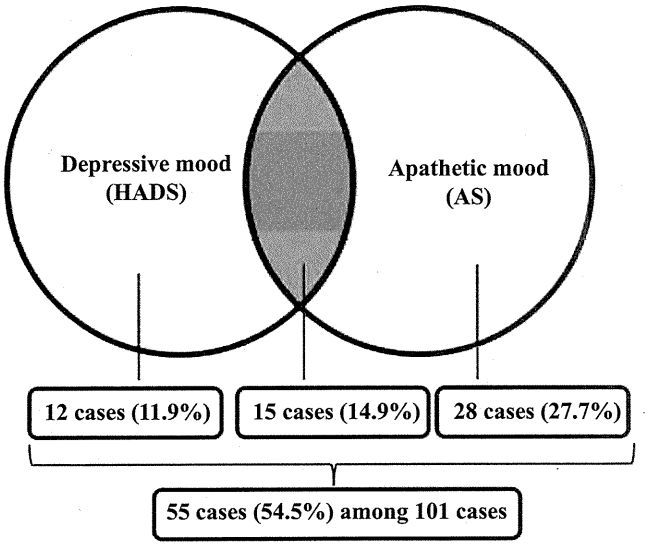

Figure 1 Incidence of post-stroke depression/apathy. The numbers show the incidence of depression/apathy in a sample of 101 stroke patients from Hibino Hospital, determined using the Hospital Depression and Anxiety Scale (HADS) to measure depression and the Apathy Score (AS) to measure apathy. (Reproduced with permission from Hama.[7])

terms of dependency. The maximum total FIM score is 126 and the lower the score, the greater the disability.[23,24] Post hoc tests and multiple regression analyses were used to examine the independent effects of PSD and apathy on functional outcome. Cognitive performance (Mini-Mental State Examination (MMSE) scores) and AS scores were found to be negatively correlated with FIM improvement, whereas no correlation was found between SDS scores and functional recovery.[22] Therefore, apathy may be associated with more functional ability and more likely to impact on the recovery process after stroke than depression.

Cognitive disturbance is a common consequence of stroke that can result in delayed functional recovery. Cognitive disturbance is also said to be a relatively common symptom of PSD.[25] The possible relationships between PSD and/or apathy and cognitive disturbance have been examined.[7] Depression was examined using the HADS (cut-off point = 8) and apathy was examined using the AS (cut-off point = 16). Memory functioning was examined using the Japanese version of the Rivermead Behavioral Memory Test, and attention was examined using the Clinical Assessment for Attention (CAT). It is known that CAT scores differ as a function of age and, to account for any age differences, we calculated corrected values as follows: (CAT score)/(age-specific mean CAT value); the lower the corrected score, the greater the attentional disturbance. There was no significant age difference between depressed and non-depressed or apathetic and non-apathetic groups. A Mann-Whitney test showed that depressed or apathetic patients had significantly lower memory and attention scores (Fig. 2). Depression and apathy appear to be associated with reduced attention and memory performance.[7] These cognitive disturbances may, in turn, be associated with impairments in functional recovery after stroke.

LESION LOCATION AND PSD/APATHY

Many previous studies of major depression in stroke patients have demonstrated morphological changes with respect to the left frontal lobe, left basal ganglia, right hemisphere, and medial orbital frontal cortex, with some suggestion that the anatomical correlates of PSD can change over time (Fig. 3a).[2,7,11,26–36] Conversely, other studies have reported no correlation between PSD and lesion location (Fig. 3a).[7,37–41] This inconsistency has precluded a clear neuroanatomical model of mood regulation after stroke, at least until recently. However, previous studies did not specifically address the two distinct dimensions of PSD, namely depressed mood (affective PSD) and loss of interest (apathetic PSD), which also comprise the two core symptoms of a major depressive episode according to DSM-IV criteria. We examined these affective and apathetic dimensions of PSD separately using the SDS and AS, respectively, and investigated possible correlations between these dimensions and damage to the basal ganglia or frontal lobe (individuals were classified into four groups: (i) no damage; (ii) damage to the left side only; (iii) damage to the right side only; and (iv) damage to both sides).[20] We found that the severity of affective depression was related to left frontal lobe damage but not to basal ganglia damage.[20] Apathetic depression was not related to frontal lobe damage, but was related to basal ganglia damage in both the right and left hemispheres.[20] These findings are consistent with previous demonstrations that depressive dimension is associated with left frontal lobe functioning (the so-called left frontal lobe theory),[10,11] whereas the apathetic dimension is associated with the basal ganglia (Fig. 3b).[4,6,42,43] The two separate core symptoms in PSD (depressed

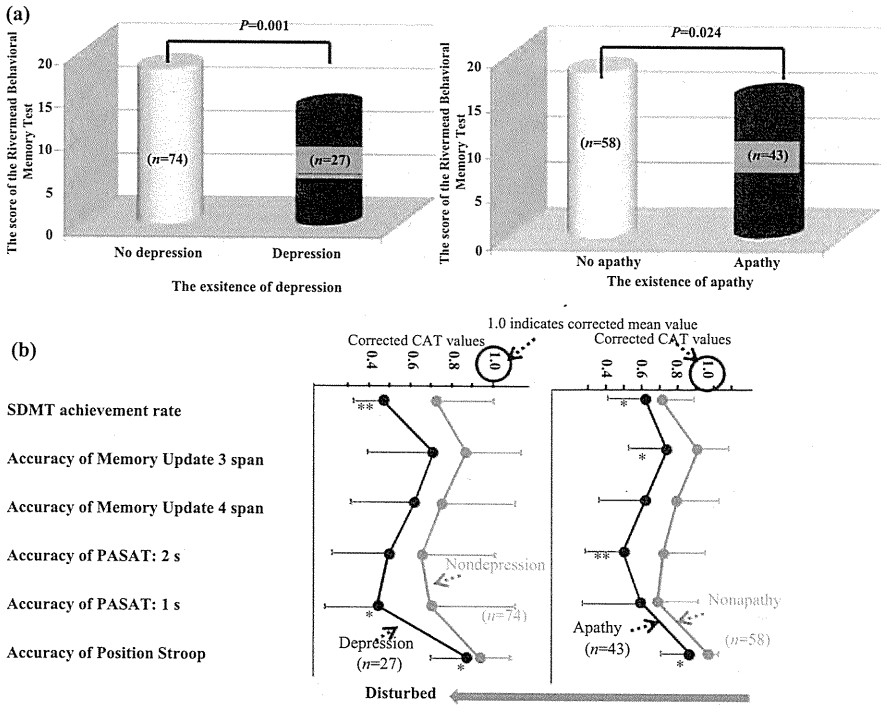

Figure 2 Correlations between post-stroke depression/apathy and cognitive functioning. Depression was examined using the Hospital Depression and Anxiety Scale (HADS) and apathy was examined using the Apathy Scale (AS). (a) Memory functioning was examined using the Japanese version of the Rivermead Behavioral Memory Test and (b) attention was examined using the Clinical Assessment for Attention (CAT). To account for age differences in CAT scores, corrected CAT values were calculated as (CAT score)/(age-specific mean CAT value); the lower the corrected score, the greater the attentional disturbance (see text for details). SDMT, Symbol Digit Modalities Test, PASAT, Paced Auditory Serial Addition Test. (Reproduced with permission from Hama.[7])

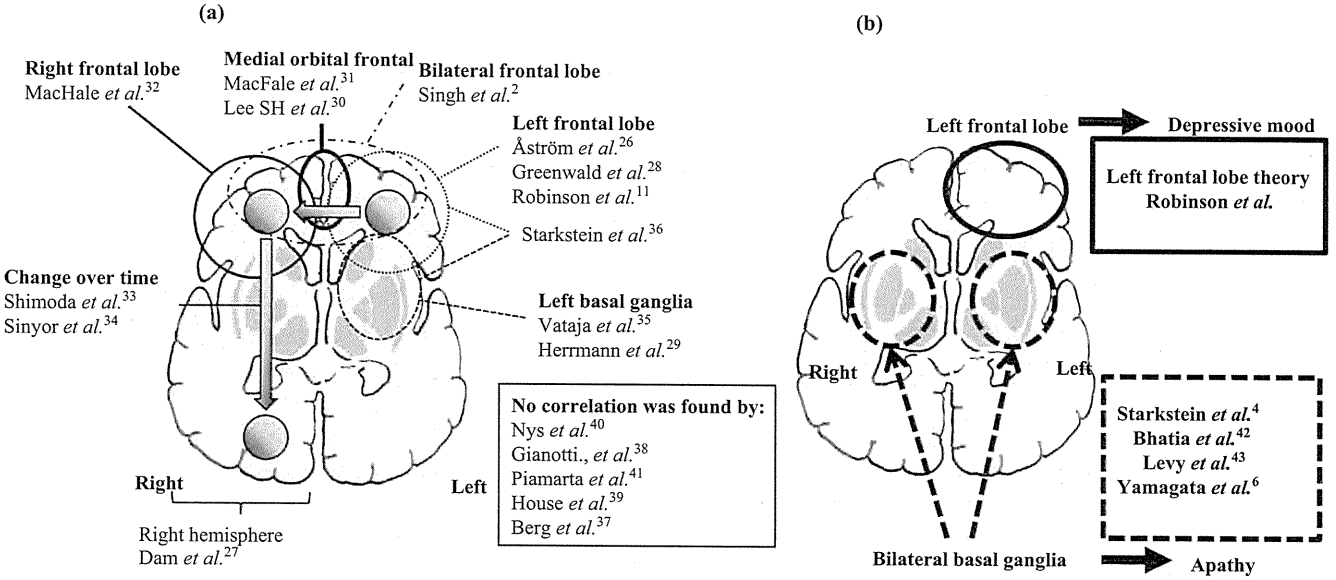

Figure 3 Schematic diagram of correlations between lesion location and post-stroke depression. (a) Summary of findings from previous studies and (b) findings of our previous study.[20] (Reproduced with permission from Hama.[7])

mood or loss of interest) clearly have different underlying neuroanatomical mechanisms.

SCHEMATIC DIAGRAM OF PSD

As noted above, PSD appears to consist of two dimensions: affective (depressive) and apathetic (Fig. 4).[7] These two core symptoms have different underlying neuroanatomical mechanisms and appear to exert different effects on functional recovery. To help patients gain increased independence, future studies should examine whether these differences in lesion location and influence on functional recovery

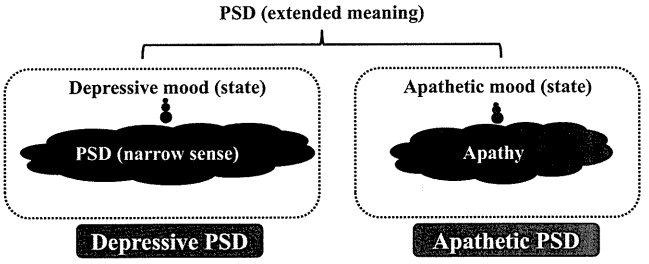

Figure 4 Schematic diagram of two core symptoms of post-stroke depression (PSD): depressive and apathetic. These two core symptoms are thought to be associated with different underlying neuroanatomical mechanisms. (Reproduced with permission from Hama.[7])

may also be reflected in different patterns of treatment response, separately or together.

It should be noted that some degree of depressive affect is thought to be a normal emotional response to newly acquired disabilities. The distinction between normative depression or apathy and 'pathological depression' or depressive illness must be made carefully.

EMOTIONAL RESPONSES AFTER STROKE: INSISTENCE ON RECOVERY

In the rehabilitation field, the prevailing view is that successful engagement in rehabilitation requires some acceptance of one's disability. However, depressed patients often struggle to make important decisions, and acceptance of a disability could be considered just such a decision. Therefore, interventions that foster increased acceptance may aid rehabilitation efforts. In this section, we focus on emotional responses after stroke.

Emotional responses to stroke have traditionally been thought to follow a mourning process that moves towards ultimate acceptance of disability.[8,9] Disabled stroke patients who refuse to accept their impairment eventually do realize that they have disabilities, but continue to think that they will soon overcome these and get well.[8,9,44,45] They manifest this attitude through direct verbal reports, or the attitude is inferred from behavior (we term this cognitive/emotional state 'insistence on recovery').[46] It was traditionally thought that patients who insist on recovery can be motivated to do any work perceived as aiding recovery, but are not motivated to learn to function as a disabled person and so often fail to gain the maximum benefit from rehabilitation services.[8,9,44,45] Thus, insistence on recovery is often considered to be maladaptive and is thought to be an appropriate target for psychotherapeutic intervention. However, the nature of insistence on recovery is just beginning to be clarified.[8,9,44–46] We examined the effect of insistence on recovery on functional independence in stroke patients, including those suffering from depression and apathy.[46]

Insistence on recovery was defined on the basis of patients' direct verbal reports or was inferred from their behavior, such that an insistence on recovery score was generated using a scale on which complaints were noted rarely, some of the time, much of the time, or most of the time (scores of 1, 2, 3, and 4, respectively).[46] These complaints suggested that the patients were preoccupied with their physical condition and were apt to overestimate the meaning of any small improvements. Appropriate levels of insistence on recovery were associated with reduced depression and apathy (Fig. 5).[46] Moreover, the effect of insistence on recovery on functional improvement was examined using multiple regression, which indicated that insistence on recovery scores were a good predictor of FIM improvement (FIM gain per week), particularly in elderly patients.[46]

Once a stroke survivor has incurred stroke-related disabilities, changes in physical functioning must be incorporated into a revised self-image, along with accompanying changes in personal values and lifestyle. This process has been dubbed 'identity crisis'.[47–49] Coping with this identity crisis is a painful process that sometimes results in depression.[46,48] During such occasions, disabled stroke patients may experience two opposing feelings, one positive (beliefs about restoration of functioning, maintenance of hope) and one negative (realizing that the disability will be permanent). A mild level of insistence on recovery is thought to be associated with certain positive feelings (e.g. hope) and may serve to minimize the seriousness of the identity crisis, reducing the pain experienced during the process of defining the disability.[46,48] Many stroke patients confront this painful mourning process (defining their disability) gradually in order to keep depressive or apathetic symptoms to a minimum, easing emotional pain with the help of a more optimistic mindset.[46] Conversely, we also found that a severe or very high level of insistence on recovery, which occurs in a limited number of cases, constitutes an unhelpful irrational belief that can lead

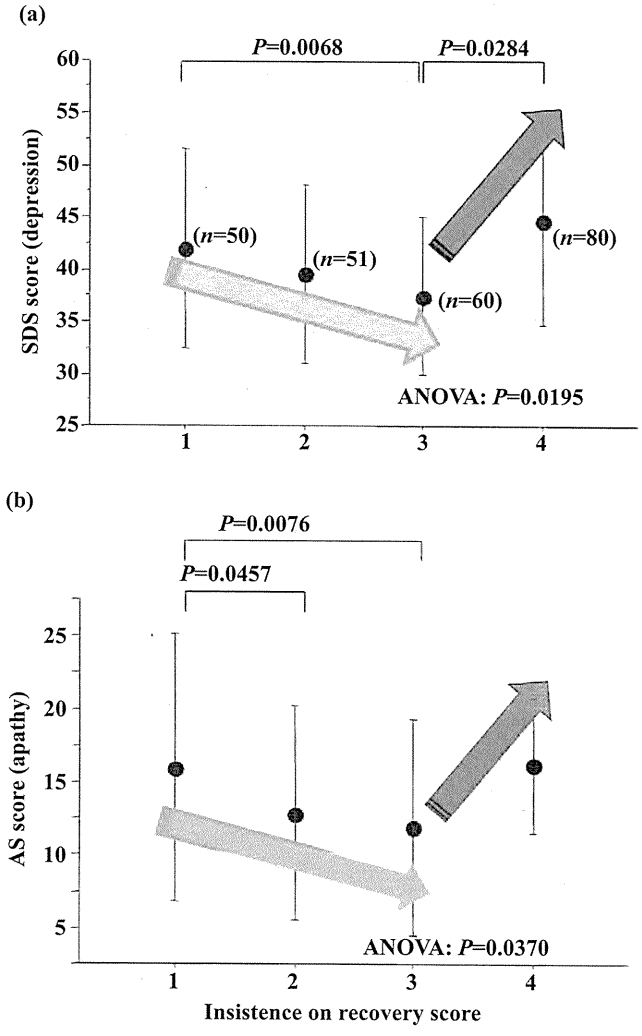

Figure 5 Differences in (a) Zung Self-rating Depression Scale (SDS) and (b) Apathy Scale (AS) scores as a function of insistence on recovery scores, generated using a scale on which complaints were noted rarely, some of the time, much of the time, or most of the time (scores of 1, 2, 3, and 4, respectively; see text for details). The data are the mean with 95% confidence intervals. Fisher's protected least significant difference test also indicated that these parameters could distinguish between these psychological subgroups. Appropriate levels of insistence on recovery were associated with reduced post-stroke depression/apathy, but extreme levels of insistence on recovery were associated with greater levels of both symptoms. The arrows indicates that the AS or SDS score changed over the elevation of the score of "Insistence on recovery". (Reproduced with permission from Hama *et al.*[46])

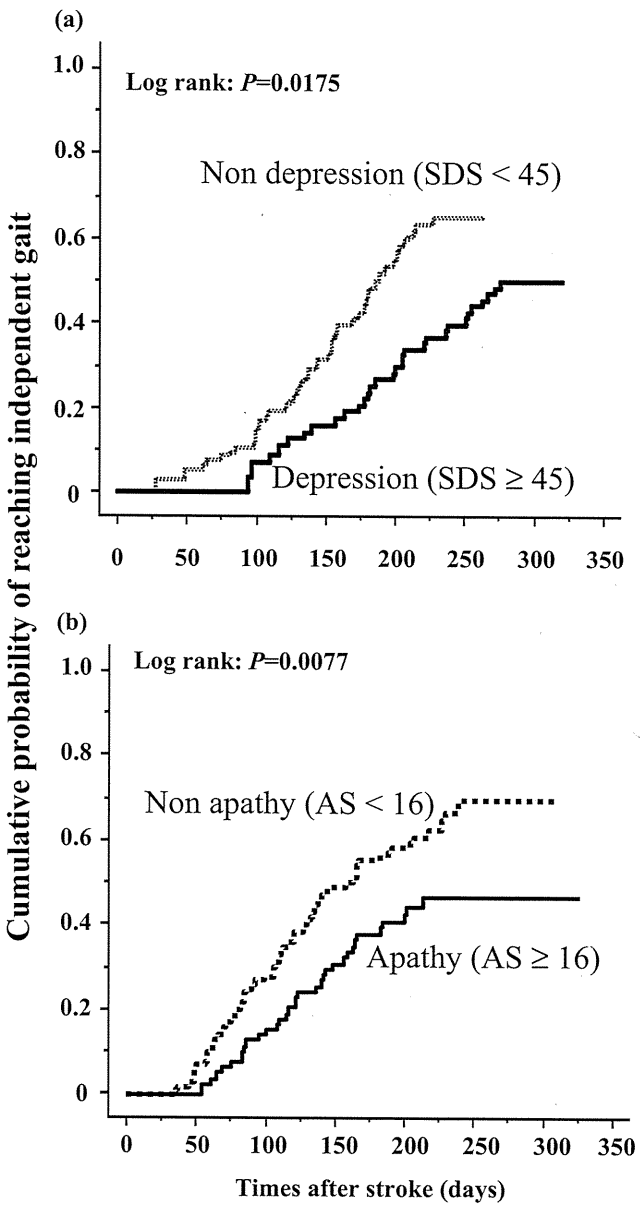

Figure 6 Survival analysis of time to achieve independent walking. Differences between (a) depression and no depression and (b) apathy and no apathy are shown as log rank *P* values. SDS, Zung Self-rating Depression Scale; AS, Apathy Scale.

patients to develop depressive symptoms and to show reduced levels of functional improvement during rehabilitation.[46,50–52] Although patients with severe levels of insistence on recovery are in the distinct minority, their characteristics are conspicuous, which can lead staff to believe that any level of insistence on recovery prevents improvement, irrespective of the degree of severity.[46] This would be problematic, because it is possible that moderate levels of insistence on recovery are, in fact, a favorable prognostic factor for disabled stroke patients.

In a previous study, we asked patients about their hopes for recovery and few of them emphasized a need to attain full recovery. Most patients shared reasonable and realistic hopes or plans. Indeed, most patients do eventually confront their own disabilities