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Neuropeptide Y Y_1 and Y_2 Receptor mRNA Expression in the Prefrontal Cortex of Psychiatric Subjects

Relationship of Y_2 Subtype to Suicidal Behavior

Laura Caberlotto, Ph.D., and Yasmin L. Hurd, Ph.D.

It has been hypothesized that the neuropeptide Y (NPY) system is involved in the pathogenesis of mood disorder. In this study, Y_1 and Y_2 receptor mRNA expression levels were analyzed in the dorsolateral prefrontal cortex of subjects affected with major depression, bipolar disorder, or schizophrenia and compared to normal controls. No significant alterations in Y_1 or Y_2 mRNA expression levels were observed between the groups. However, the Y_2 mRNA expression was elevated in layer IV in subjects with suicide as a cause of death. For the Y_1 mRNA expression, there was a negative correlation with increasing subject age in the prefrontal cortex. Analysis of covariance revealed a significant elevation of the Y_1 mRNA expression levels in individuals with a current history of marijuana use but no other drug. In summary, the current results suggest distinct alterations of the prefrontal Y_1 and Y_2 neuronal populations in aging and suicide.

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KEY WORDS: *Neuropeptide Y receptors; Post-mortem; Affective disorder; Schizophrenia; Aging; Suicide*

Numerous attempts have been made to understand the pathogenesis of affective disorders in terms of altered neurotransmitters. Monoamines have received the main focus in this line of research, but neuropeptides such as somatostatin (Rubinow 1986), corticotrophin-releasing factor (Nemeroff et al. 1984), and endogenous opioids (Gross-Isseroff et al. 1990; Scarone et al. 1990) have also been studied in relation to depressive disorders.

The involvement of neuropeptide Y (NPY), a 36 amino acid neurotransmitter member of the pancreatic polypeptide family, in the pathophysiology of affective disorders has been supported by copious evidence suggesting an impairment of the NPY system in depression and bipolar disorders, although some contrasting results have also been reported. NPY-LI was found to be reduced in the cerebrospinal fluid (CSF) (Widerlöv et al. 1988) and plasma (Hashimoto et al. 1996; Nilsson et al. 1996) of depressed subjects, and in the frontal cortex and caudate nucleus of suicide victims, particularly in a subgroup of subjects affected by major depression (Widdowson et al. 1992). In addition, a reduction of NPY mRNA was observed in the prefrontal cortex of subjects diagnosed with bipolar disorder (Caberlotto and Hurd 1999). Various antidepressant treatments have been demonstrated to increase NPY levels in the CSF of humans (Mathé et al. 1996) and in specific regions of the rat brain (Mathé et al. 1990; Stenfors et al. 1989; Wahlestedt et al. 1990; Weiner et al. 1992; Zachrisson et al. 1995).

NPY effects are mediated through at least five distinct receptor subtypes (Michel et al. 1998). Of these, the

From the Karolinska Institute, Department of Clinical Neuroscience, Psychiatry Section, Karolinska Hospital, Stockholm, Sweden.

Address correspondence to: Dr. Yasmin Hurd, Department of Clinical Neuroscience, Psychiatry Section, Karolinska Hospital, S-171 76, Stockholm, Sweden. Tel: 46-8-5177-2379; Fax: 46-8-346563. E-mail: Yasmin.Hurd@ks.se

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Table 1. Demographic Information Obtained from the Stanley Foundation Neuropathology Consortium on the Brain Specimens Examined

	Control	Major depression	Bipolar disorder	Schizophrenia
Age	48.1 (29–68)	46.5 (30–65)	42.3 (25–61)	43.6(25–62)
Sex	9M, 6F	9M, 6F	9M, 6F	9M, 5F
Race	14W, 1B	15W	14W, 1B	12W, 2As
PMI (hours)	23.7 (8–42)	27.5 (7–47)	32.5 (13–62)	34.2 (12–61)
pH	6.3 (5.8–6.6)	6.2 (5.6–6.5)	6.1 (5.8–6.5)	6.1 (5.8–6.6)
Side of brain	7R, 8L	6R, 9L	8R, 9L	6R, 9L

M, male; F, female; W, white; B, black; As, asian; R, right, L, left.
Range shown in parentheses.

Y_1 and Y_2 receptors are the most abundant in the brain. We had previously hypothesized a role of the Y_1 receptor in affective disorders based on data obtained from an animal model of depression, the Flinders Sensitive Line rats (Caberlotto et al. 1998). In these rats, the Y_1 receptor mRNA expression levels were decreased in specific limbic and cortical regions. In contrast, the Y_2 receptor mRNA expression was not altered in the Flinders Sensitive Line rats. Although these results suggest a potential involvement of the Y_1 in depression, no information is currently available on the possible alteration of the NPY receptors in human subjects diagnosed with affective disorders or other psychiatric disorders.

Based on our previous finding of reduced NPY mRNA expression in the prefrontal cortex of bipolar subjects (Caberlotto and Hurd 1999), it was of interest to determine whether the NPY receptor genes were also impaired in subjects with mood disorders. To that end, the mRNA expression levels of Y_1 and Y_2 receptor were examined in the prefrontal cortex of subjects affected by bipolar disorder, major depression, or schizophrenia, and compared with normal controls. Moreover, the effect of suicide as a cause of death was also examined in relation to the Y_1 and Y_2 mRNA expression levels.

MATERIAL AND METHODS

Prefrontal cortical specimens (Brodmann area 9 and 46; 14 μm-thick frozen sections) were obtained from the Stanley Foundation Neuropathology Consortium that collected the brains under approved ethical guidelines. Four groups were studied: schizophrenia, bipolar disorder, major depression without psychotic feature, and normal control. The demographic information is presented in Table 1. Psychiatric diagnosis was established independently by two senior psychiatrists using DSM-IV criteria based on information obtained from, for example, hospital records, pathologists, and/or interviews with family members or treating professionals (see Torrey et al. 2000). The groups had been matched for age, sex, post-mortem interval (PMI), and brain hemisphere. The brains studied had also been matched for mRNA stability (GAPdH and actin) and for pH (Table 1). All demographic information and documented medical data (e.g. lifetime fluphenazine antipsychotic treatment) about the subjects were provided by the Stanley Foundation Neuropathology Consortium. Information was also provided as to the substance abuse history. Subjects with "current drug use" are defined

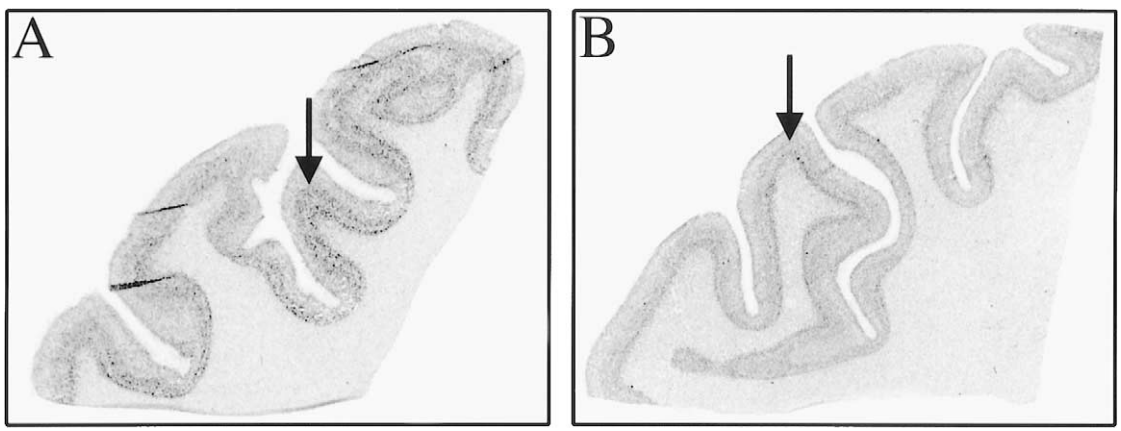

Figure 1. Y1 mRNA expression in the human prefrontal cortex of (**A**) a young (25 years of age) and (**B**) an older (53 years of age) subject. Note the reduction of the Y1 mRNA expression in the cortex of the older subject, particularly in layer IV. Arrows point to layer IV.

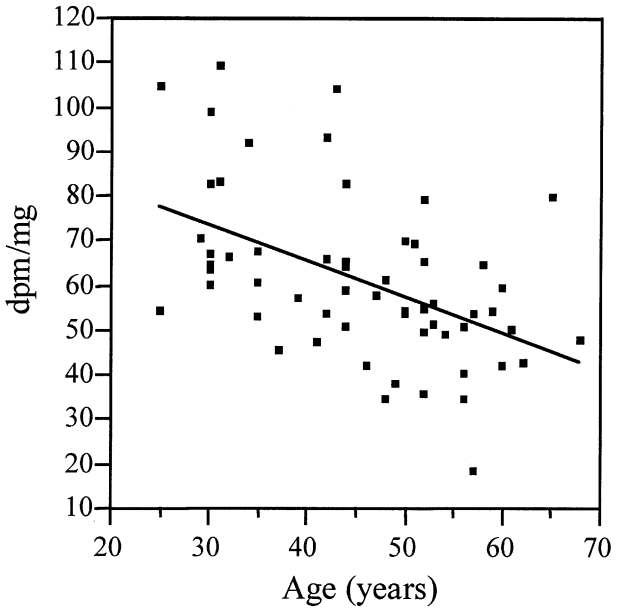

Figure 2. Linear regression line of the Y1 mRNA expression in layer IV of the prefrontal cortex with increasing age. A significant negative correlation ($r = -0.4939$; $p = .0001$) between Y_1 mRNA expression levels in layer IV and age was observed.

based on a documented history of drug use, abuse, or dependence diagnosis, "past use" defined as prior but not present drug use, and "no use" defined as no current or past drug use history.

Probes Preparation

The Y_1 probes were made from a 0.4 Kb cDNA fragment of the human Y_1 receptor (Larhammar et al. 1992), corresponding to amino acids 1-145 of the protein, which had been subcloned in a pGEM4Z vector (courtesy of GlaxoWellcome). A 629 bp Fsp/HincII fragment of the human Y_2 cDNA (Rimland et al. 1996; courtesy of GlaxoWellcome), that spans the coding region of the receptor from the second to the sixth transmembrane domains, was subcloned in a PBKSII vector and used to generate RNA probes. Before transcription, these plasmids were linearized with the appropriate restriction enzymes for generating the antisense and sense riboprobes. RNA probes complementary to the coding sequences were transcribed from the linearized plasmid template with 150 μCi α-[^{35}S]UTP (Dupont NEN, Boston, USA). T3, T7, or SP6 RNA polymerases were used for generating the antisense and sense probes. Transcription occurred in the presence of 10 mM dithiothreitol, 0.5 mM each of ATP, GTP, CTP, and 1 μg linearized plasmid template in a 1X transcription buffer for 60 min at 37°C. The labeled probes were then separated from unincorporated nucleotides using spin columns (Pharmacia Biotech, Stockholm, Sweden).

In Situ Hybridization

Before hybridization, the brain sections were warmed to room temperature and allowed to dry. Subsequently, the sections were fixed in 4% paraformaldehyde/1X phosphate buffered saline (PBS) for 5 min, rinsed twice in 1X PBS, and treated with 0.25% acetic anhydride/0.1 M triethanolamine/0.9% sodium chloride for 10 min. The sections were then rinsed in 2X standard saline citrate (SSC; 1 × SSC = sodium chloride 0.15 M, sodium citrate 0.015 M), dehydrated in a graded series of ethanol (70, 80, 95, 100%), delipidated with chloroform, and air dried before the hybridization procedure. All aqueous solutions were pretreated with 0.1% diethylpyrocarbonate before use. The hybridization buffer consisted of 0.5 mg/ml sheared ss DNA, 250 μg/ml yeast tRNA, 1X Denhardt's solution (solution of 0.2% each, bovine serum albumin, ficoll, polyvinylpyrrolidone), 10% (w/v) dextran sulfate, 4X SSC, and 50% formamide. Before hybridization, the labeled probe was added to the hybridization cocktail in a concentration of 20×10^3 cpm per μl, and 0.2 ml of this hybridization mixture was applied to the brain sections. The sections were coverslipped, and the hybridization was carried out in a humidified chamber overnight at 55°C. Incubation was followed by washes in a graded series of SSC solutions (2X SSC, 2 × 5 min; 1X SSC and 0.5X SSC, 10 min; 0.1X SSC, 1 h) containing 1 mM dithiothreitol, all at room temperature except for the 0.1X SSC (53°C). De-

Table 2. Y_1 and Y_2 mRNA Expression Levels (Mean ± S.E.M. expressed in dpm/mg) in Different Layers of the Prefrontal Cortex in the Four Diagnostic Groups

	Control	Major depression	Bipolar disorder	Schizophrenia
Y1 all layers	53.45 ± 7.53	58.79 ± 21.28	53.08 ± 15.34	55.68 ± 16.40
Y1 sup. layers[a]	44.96 ± 19.79	44.75 ± 17.29	38.57 ± 14.50	37.83 ± 13.91
Y1 layer IV	58.50 ± 15.27	65.50 ± 21.11	63.40 ± 18.45	59.90 ± 19.58
Y1 deep layers	71.98 ± 20.24	75.69 ± 27.13	65.52 ± 18.60	65.35 ± 22.66
Y2 all layers	16.44 ± 6.70	14.20 ± 6.00	12.80 ± 8.44	11.39 ± 6.68
Y2 layer IV	27.24 ± 6.75	24.94 ± 7.16	21.68 ± 10.55	21.26 ± 8.16

[a] $p < .05$ main group effect (ANCOVA), but Tukey–Kramer post-hoc comparison revealed no significant differences between any of the groups.

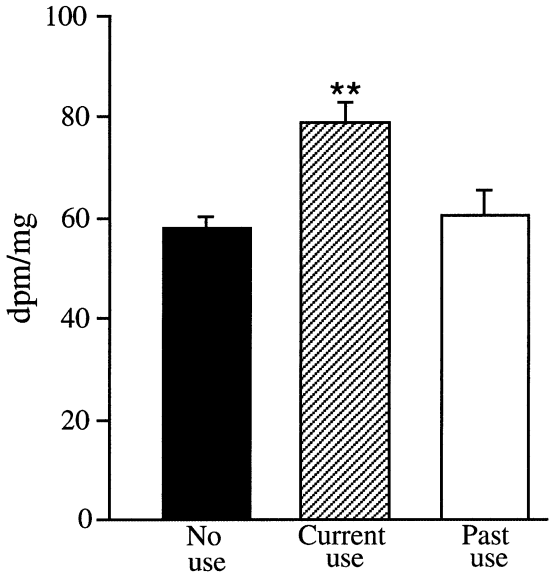

Figure 3. Y1 mRNA expression levels (dpm/mg) in layer IV of the prefrontal cortex in relation to the history of marijuana use. No use (n = 43; age = 47.42 ± 1.67 years old); current use (n = 6; age = 36.30 ± 4.18 years old); past use (n = 8; age = 41.0 ± 3.71 years old). A significant over-all group difference was observed ($F_{2, 47}$ = 3.7294, p = .0314; ANCOVA). **p = .01 Tukey–Kramer post-hoc comparison versus no use and current use.

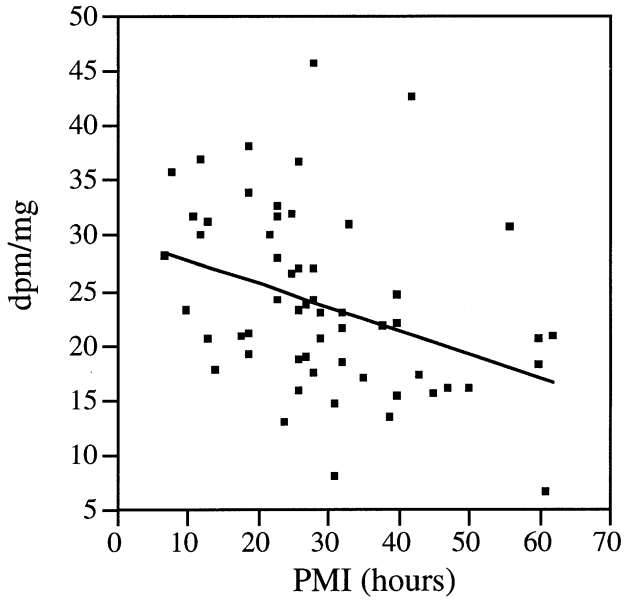

Figure 4. Linear regression line of the Y_2 mRNA expression in layer IV of the prefrontal cortex and PMI. A significant negative correlation (r = −0.3476; p = .0075) between Y_2 mRNA expression levels in layer IV and PMI was observed.

hydration was carried out with graded ethanol solutions containing 300 mM ammonium acetate. The slides were then air dried and exposed to Hyperfilm (Amersham, Buckinghamshire, England) along with ^{14}C standards for a period of 2 to 3 weeks.

Film autoradiograms were scanned at a resolution of 250 dpi with a ScanMaker III (Microtek Electronics, Düsseldorf, Germany). Light transmittance values were measured from the digitalized images with a Macintosh-based image analysis software system (NIH Image, Wayne Rasband, NIMH). Two slides per subjects were measured. A minimum of six measurements were taken of each cortical slide and averaged. Background signal in the adjacent white matter was subtracted from the averaged values. In addition to the total laminar measurement, transmittance values were taken within superficial layers, layer IV, and deep layers for the Y_1 and within layer IV for the Y_2 considering the distinct expression patterns of the respective NPY receptor hybridization signals in the neocortex. Based on the known radioactivity in the ^{14}C standards relative to their transmittance levels, the light transmittance values were converted to dpm/mg using a Rodbard calibration curve (NIH Image).

Statistical Analysis

Analysis of covariance (ANCOVA) was used to determine group differences in dpm/mg (mRNA expression) levels measured in the cortical layers examined. Independent variables (age, PMI, sex, hemisphere side, and documented history of stimulant (cocaine/amphetamine), marijuana, or alcohol use) were included in the statistical model if results from univariate analysis showed a p-value of $< .250$ (Bendel and Afifi 1977) for that variable. Significant (p = .05) differences obtained from the ANCOVA analysis were further assessed by Tukey–Kramer post-hoc comparison. The influence of suicide as a cause of death, age of disease onset, and duration of the disease on mRNA expression levels was determined only in the psychiatric groups. Pearson Correlation analysis was also performed. All the statistical evaluations were performed using the JMP (3.1v) statistical software package.

RESULTS

Y_1 mRNA Expression

The Y_1 receptor mRNA distribution pattern (Figure 1) was consistent with that previously described (Caberlotto et al. 1997). Hybridization signals were observed throughout all cortical layers with the highest labeling in layers IV and VI. A fair to moderate relationship was found between the dpm/mg values and age: there was a negative correlation between Y_1 mRNA expression levels and increasing age (all layers, r = −0.3542, p = .0059; superficial layers, r = −0.3785, p = .0031; layer

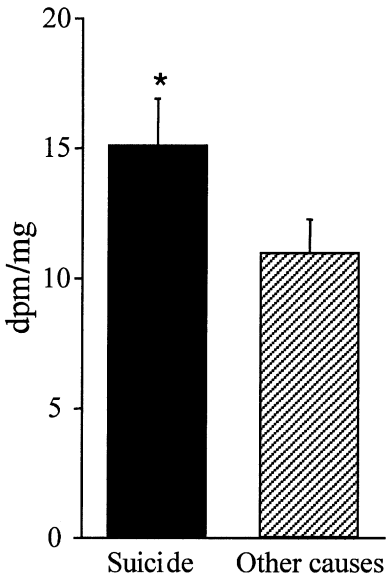

Figure 5. Y2 mRNA expression levels (dpm/mg) in layer IV of the prefrontal cortex in relation to cause of death. A significant increase of the Y_2 mRNA expression was observed in suicide victims (*p = .0181; ANCOVA). Suicide, n = 20; PMI = 30.45 ± 3.5 hours. Non-suicide, n = 39; PMI = 28.2 ± 1.98 hours.

IV, r = −0.4939, $p < .0001$; Figures 1 and 2). As such, age was included in all ANCOVA analyses. The residual plots for all analyses were normally distributed. There was no significant effect of PMI, hemisphere, or sex on the dpm/mg levels. However, in the superficial layers, there was an over-all significant group effect ($F_{3, 48}$ = 2.9089, p = .044; Table 2), but post-hoc analysis revealed no significant differences between each group. Subjects with suicide as a cause of death tended to have higher Y_1 mRNA expression levels, but these individuals were among the youngest (< 45 years old) in the population studied. Overall, there was no significant effect of suicide as a cause of death on the Y_1 mRNA expression levels with the ANCOVA analysis. In addition, no significant correlation was found in regard to the age of disease onset and the duration of the disease for the Y_1 mRNA expression levels detected in the psychiatric groups.

No significance was observed in relation to the dpm/mg values and the lifetime antipsychotic treatment (fluphenazine) in the schizophrenic and bipolar disorder groups. Moreover, there was no significant effect of the history of stimulant (cocaine/amphetamine) or alcohol use in the subjects. However, a significant influence was found regarding the history of marijuana use (ANCOVA: all layers, $F_{2, 47}$ = 3.8725, p = .0277; superficial layers $F_{2, 48}$ = 5.2210, p = .0089; layer IV, $F_{2, 47}$ = 3.7294, p = .0314). Post-hoc analysis revealed that subjects with a recent history of marijuana use had higher Y_1 mRNA expression levels (in the total of all layers and in layer IV) compared with nonusers (Figure 3). Although subjects with current marijuana use were on average younger (36.30 ± 4.18 years) than non-users (47.42 ± 1.67 years), there was no significant age by marijuana interaction ($F_{2, 51}$ = 1.9871, p = .1476).

Y_2 mRNA Expression

Hybridization with the Y_2 riboprobe produced a characteristic pattern consistent with previous findings (Caberlotto et al. 1998) with highest intensity in layer IV of the prefrontal cortex. Y_2 mRNA expression levels were found to be normally distributed. No significant correlation was found between the Y_2 mRNA expression levels measured in the prefrontal cortex and subject age. However, a negative correlation was detected between PMI and Y_2 mRNA expression in the total cortical area (r = −0.2662, p = .0434) and layer IV (r = −0.3476, p = .0075; Figure 4). PMI was included in the ANCOVA models. No significant differences in the Y_2 mRNA expression levels were detected between the diagnostic groups (Table 2), sex, hemisphere, or the documented use of alcohol, marijuana, or stimulants. There was, however, a significant effect of suicide as a cause of death in the psychiatric groups ($F_{1, 39}$ = 6.0886, p = .0181), but there was no suicide by diagnosis interaction ($F_{2, 38}$ = 1.5171, p = .2323). Subjects committing suicide showed higher Y_2 mRNA expression levels in layer IV (Figure 5). A similar trend was also evident in the total cortical area ($F_{1, 40}$ = 3.5428, p = .0671). The age of onset and the duration of the disease had no significant influence on the mRNA expression in the psychiatric groups. In addition, no significance was found in relation to the lifetime fluphenazine treatment in the schizophrenia and bipolar subjects.

DISCUSSION

The present study revealed a progressive age-related decline in the expression of the Y_1 receptor mRNA in the human prefrontal cortex. The decrease of Y_1 mRNA expression with increasing age could reflect a general loss of cortical cells that normally occurs in aging (DeKosky and Bass 1982). However, other mRNAs (e.g., prodynorphin and kappa opiate receptor, Peckys and Hurd unpublished data) that were studied in the same cortical specimens were not affected by age. It could also be speculated that the current results reflect a nonspecific loss of NPY-related neurons. Some experimental animal studies have shown impairment of NPY cortical neurons with increasing age (Cha et al. 1997; Zhang et al. 1998). However, unlike the Y_1 receptor mRNA expression levels, neither NPY (Caberlotto and Hurd 1999) nor the Y_2 (present study) mRNA expression levels showed a similar sensitivity to age in the

brain specimens currently examined. In the human neocortex, we have observed that the Y_1 mRNA lacks coexpression with NPY neurons; whereas, the Y_2 is highly colocalized (40%) to NPY cells (Caberlotto and Hurd in press). It is possible that there is a selective sensitivity of cortical neurons expressing the Y_1 gene during aging.

We previously observed a reduction of NPY mRNA expression in bipolar subjects, but none of the psychiatric groups currently studied differed from the normal subjects in regard to the expression of either the Y_1 or Y_2 mRNAs. The Y_2 receptor mRNA was, however, significantly altered in suicide victims. This is the first report of an impairment of the prefrontal cortex NPY receptor mRNA expression with suicide, a cause of death highly related with mood dysfunction. It is estimated that over 90% of suicide victims have psychiatric disorders: 50–70% affective disorders, 8–12% schizophrenia, and 8–10% substance use disorder (Rihmer 1996). In the population presently studied, individuals with suicide as a cause of death tended to have higher Y_1 mRNA expression levels, but a large number of the psychiatric subjects who committed suicide were young. Given the impact of age on the Y_1 mRNA levels, no significant suicide effect was observed on the Y_1 mRNA expression in the ANCOVA analysis. A clear significant increase of the Y_2 receptor mRNA expression, which was not influenced by subject age, was detected in the prefrontal cortex in suicide victims regardless of their psychiatric diagnosis. These findings suggest that the Y_2 mRNA alteration is not directly associated with mood disorder and that the increased prefrontal Y_2 mRNA expression is perhaps more related to other aspects of suicidal behavior than to the psychiatric condition of the subject.

Interestingly, the history of recent marijuana use was found to be associated with an increase of the Y_1 mRNA expression levels in the prefrontal cortex. It is difficult to assess fully the significance of this finding considering the limited information available about the effects of marijuana on the NPY system in the brain. In the human brain, it has been shown that cannabinoid receptor binding sites are highly localized to the frontal cortex (Glass et al. 1997). Thus, it is feasible that marijuana could influence the Y_1 receptor mRNA-expressing neurons in the prefrontal cortex. Further studies are necessary to validate the present findings in a larger population of marijuana users and to assess the possible interactions between the NPY and the cannabinoid cortical systems. In contrast to marijuana, no other drug; for example, alcohol or stimulant (cocaine/amphetamine) was found to influence the Y_1 or Y_2 mRNA expression levels. The lifetime use of the antipsychotic drug fluphenazine was also not significantly correlated to the Y_1 or Y_2 mRNA expression levels in the prefrontal cortex. Fluphenazine was the only medication included in the current analysis, because information regarding detailed toxicology and the history of other antipsychotics or antidepressant medications was not available. The lack of such information is one limitation of the present study, and follow-up investigations are needed to address this issue.

A number of post-mortem parameters can affect the stability of mRNAs and proteins in the human brain. In this study, the Y_2 mRNA expression was significantly influenced by post-mortem delay. Prefrontal cortical Y_2 mRNA expression was negatively correlated with PMI; whereas, the Y_1 (current date) and NPY (Caberlotto and Hurd 1999) mRNA levels were not affected by the post-mortem delay time. In a previous investigation of the effects of PMI in an animal model simulating human autopsy conditions, we demonstrated the susceptibility of Y_1 binding sites, but not of Y_1 mRNA expression levels or Y_2 binding sites, to PMI (Caberlotto et al. 1997), consistent with the current findings. The Y_2 mRNA expression was not evaluated in that study, thus the factors that differentially affect the NPY receptor mRNAs and protein stability call for further investigation.

In conclusion, the present results reveal an age-related decline of the Y_1 mRNA expression in the prefrontal cortex. In addition, there are indices of an impairment of the prefrontal cortical NPY receptor mRNA expression in suicide, but this might not relate to the pathophysiology of mood disorder.

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