Brain Research Bulletin, Vol. 55, No. 5, pp. 619–624, 2001

0361-9230/01/$–see front matter

PII S0361-9230(01)00525-1

Prodynorphin and κ opioid receptor mRNA expression in the cingulate and prefrontal cortices of subjects diagnosed with schizophrenia or affective disorders

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ABSTRACT: The present study examined the prodynorphin and κ opioid receptor mRNA expression levels in the anterior cingulate and dorsolateral prefrontal cortices of subjects diagnosed with schizophrenia, bipolar disorder, or major depression as compared with normal controls without a psychiatric diagnosis. Multivariate analyses failed to reveal any differences in the mRNA expression levels between the four diagnostic groups, though a group trend (non-significant) was evident for the expression of the κ opioid receptor and prodynorphin mRNAs in the prefrontal cortex. The mRNA expression levels were not associated with lifetime history of antipsychotic treatment or with suicide as a cause of death. The results, however, suggested an influence of certain drugs of abuse on the prodynorphin cortical mRNA expression. Prodynorphin mRNA expression levels were found to be elevated in individuals with a history of marihuana or stimulant use, but not alcohol. Overall, our data do not provide strong evidence for impaired prodynorphin or κ opioid receptor mRNA levels in the dorsolateral prefrontal or cingulate cortices of schizophrenic, bipolar disorder, or major depressed subjects. © 2001 Elsevier Science Inc.

KEY WORDS: Opioid neuropeptide, Major depression, Bipolar disorder, Psychostimulants, *In situ* hybridization.

INTRODUCTION

Dynorphin opioid neuropeptides and their main receptors, the κ opioid receptors, exhibit a widespread distribution throughout the mammalian brain [10,15,18,26,27,30,32,35,46,54,60]. This opioid neuropeptide system has been implicated in the control of motor, endocrine, and cognitive functions and is intricately linked with dopamine (DA) and serotonin (5-HT) neurotransmitters, which are believed to play a strong role in schizophrenia and depression disorders. Catecholaminergic neurons send terminal projections to dynorphin-containing neuronal populations [56] and influence the activity of the opioid cells. DA agonists increase dynorphin gene expression [17,19,24,41] and protein [41,45,47,59] levels and dynorphin and other κ agonists in their turn decrease DA release [13,49]. It has also been demonstrated that destruction of the raphe nucleus [34], which provides the main serotoninergic innervation of the central nervous system, or pharmacological serotoninergic manipulations [16] alter gene expression in dynorphinergic neurons. Moreover, activation of the κ opioid receptors depresses evoked excitatory postsynaptic potentials of serotonergic cells in the dorsal raphe nucleus [39] and reduces the release of 5-HT [11]. In the human brain, the κ opioid receptor mRNA is expressed in dopaminergic neurons of the substantia nigra as well as in neurons of the raphe nuclei [37].

Several lines of evidence suggest that, in addition to DA and 5-HT, the endogenous dynorphin peptides and the κ opioid receptors may also play a role in the pathophysiology of mental illnesses. Initial studies carried out in the mid-1980s with healthy human volunteers reported psychomimetic and dysphoric effects upon activation of κ opioid receptors [29,38]. Moreover, drugs acting at κ opioid receptors have been shown to have strong antidepressant [7,14] and antipsychotic [43] effects.

The prefrontal cortex is an important brain area for high-level cognitive and emotional processes. In schizophrenics, a growing body of evidence has been accumulated showing neuropathological, neuropsychological, and cerebral blood flow dysfunction related to the dorsolateral prefrontal cortex [1–3,28,33,48,52]. Abnormalities have also been reported in the cingulate and prefrontal cortices in subjects with major depression and bipolar disorder [5,6,8,9,36,42,57]. Despite the implications of an involvement of the dynorphin/κ opioid receptor cortical system in psychiatric disorder, no studies have directly examined the mRNA expression of either prodynorphin or the κ opioid receptor in individuals with psychiatric illnesses. In the current study, *in situ* hybridization histochemistry was used to determine the expression levels of the κ opioid receptor and the prodynorphin mRNAs in the anterior cingulate and dorsolateral prefrontal cortices of three groups of patients diagnosed with specific psychiatric disorders and in normal control subjects.

MATERIALS AND METHODS

Dorsolateral prefrontal (Brodmann area 46 and 9) and anterior cingulate (Brodmann area 23) cortical tissues were obtained from the Stanley Foundation Neuropathology Consortium that collected the brains under approved ethical guidelines. Four groups were studied: schizophrenia ($n = 14$), bipolar disorder ($n = 15$), major depression without psychotic feature ($n = 15$), and normal controls

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($n = 15$). The schizophrenic subjects (9 males and 5 females; 12 White and 2 Asian) ranged in age from 25 to 62 years (43.6 ± 3.5) and had a postmortem interval (PMI) of 34.2 ± 4.01 (range, 12–61 h). The demographic information regarding the bipolar disorder, major depression, and normal control subjects has been reported previously [53]. Diagnoses had been established by two psychiatrists usage criteria from the *Diagnostic and Statistical Manual of Mental Disorders*, fourth edition, as described by Torrey and colleagues [53]. The brains had been matched for mRNA stability (GAPdH and actin), pH (average range, 6.1–6.3), and for hemisphere side [53]. All demographic information and documented medical data, including lifetime antipsychotic (fluphenazine) medication and history of drug abuse, about the subjects were provided by the Stanley Foundation Neuropathology Consortium. Limited toxicological information was also provided about the cases.

Riboprobe Preparation

Riboprobe protocols were used that had been previously optimized to visualize the specific prodynorphin [21] and κ opioid receptor [37] mRNA expression in the human brain. The prodynorphin mRNA probe was complementary to a 1.2-kb fragment containing most of exon 4 of the human prodynorphin cDNA ([20]; courtesy of Dr. Jim Douglas). The κ opioid receptor probe was a PCR-generated 0.6-kb fragment (bases 330–965; [37] complementary to 60% the coding region of the human κ opioid receptor cDNA [44]. The RNA probes were transcribed from their templates using SP6 and [^{35}S]-UTP (New England Nuclear, Boston, MA, USA) for the prodynorphin probe and [^{33}P]-UTP (New England Nuclear) for the κ opioid receptor probe.

In Situ *Hybridization*

Brain sections used for analysis of the prodynorphin and κ opioid receptor mRNA expression levels were studied in separate experiments. In addition, the cingulate and prefrontal cortical specimens were processed on different days. Prior to hybridization, the brain sections (two slides/subject) were warmed to room temperature and allowed to dry. Subsequently, the sections were fixed in 4% paraformaldehyde/1× phosphate buffered saline (PBS) for 5 min, rinsed twice in 1× 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 2× 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, 1× Denhardt's solution (solution of 0.2% each, bovine serum albumin, ficoll, polyvinylpyrrolidone), 10% (w/v) dextran sulfate, 4× 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 0.21 ml of this hybridization mixture was applied to the brain sections. The sections were coverslipped to prevent evaporation 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 (2× SSC, 2 × 5 min; 1× SSC and 0.5× SSC, 10 min; 0.1× SSC, 1 h) containing 1 mM dithiothreitol, all at room temperature except for the 0.1× SSC (53°C). Dehydration was carried out with graded ethanol solutions containing 300 mM ammonium acetate. The slides were then air-dried and exposed to Hyperfilm (Amersham, Buckinghamshire, UK) along with ^{14}C standards (Amersham) for a period of 3 to 6 weeks.

Quantification of Autoradiograms

The autoradiograms from the *in situ* hybridization experiments were scanned using a ScanMaker III (Mikroteck Electronics, Düsseldorf, Germany) at a resolution of 300 dpi. Light transmittance values were measured from the digitalized images with a Macintosh-based image analysis software system (NIH Image; Wayne Rasband, National Institute of Mental Health, Bethesda, MD, USA). Two slides per cortical area of each subject were measured. A minimum of four measurements were taken of the total laminae, as well as in the layers of interest, of each cortical slide and averaged. Background signal in the adjacent white matter was subtracted from the averaged values. 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

Multivariate analysis was used to determine group differences in dpm/mg (mRNA expression) levels measured in the cortical layers examined. A screening model was used because it had not been previously established which variables might influence the opioid mRNA expression levels in the cingulate and prefrontal cortices. The screening criteria for selection of the variables were based on results from univariate analysis of variance. Independent variables (age, PMI, gender, hemisphere side, and documented history of stimulant (cocaine/amphetamine), marihuana, or alcohol use) were included in the model if results from univariate analysis showed a p-value of <0.250 [4]. Significant ($p \leq 0.05$) differences were further assessed by Tukey-Kramer *post-hoc* comparison. The association between suicide as a cause of death, age of disease onset, and duration of the disease on the mRNA expression levels was determined only in the psychiatric groups. All the statistical evaluations were carried out using the JMP (3.1v) statistical software package. Trends are discussed for p-values ≤ 0.10.

RESULTS

The use of the prodynorphin and κ opioid receptor riboprobes resulted in different hybridization patterns with signals expressed only in the cortex and not in the adjacent white matter. In the prefrontal cortex, hybridizations carried out with the prodynorphin riboprobe resulted in a characteristic labeling concentrated to layer IV (Fig. 1C), whereas the κ opioid receptor probe labeled primarily the deeper layer V and VI (Fig. 1D). In the cingulate cortex both probes (though relatively weaker for the prodynorphin) produced additional labeling in all other layers with no distinct laminar pattern evident in the superficial laminae (Figs. 1A,B). The dpm/mg values measured in each cortical area are provided in Table 1.

Prodynorphin and κ opioid receptor mRNA expression levels measured in the total area of the prefrontal and cingulate cortices were found to have an approximate normal distribution (based on the Shapiro-Wilk test). A weak but significant relationship was found between PMI and the mRNA expression levels in the cingulate ($r = -0.2764$, $p = 0.0341$, for the κ opioid receptor; $r = -0.2966$, $p = 0.0225$, for prodynorphin) and prefrontal ($r = -0.2739$; $p = 0.0278$, for the prodynorphin) cortices. There was no significant association between age, gender, side of brain, and the history of alcohol use and either the prodynorphin or the κ opioid receptor mRNA expression levels. However, significance or trend effects were apparent for the history of marihuana or stimulant use. As such, PMI and the history of marihuana and/or stimulant use were included in the multivariate models.

The dpm/mg values measured in each of the four diagnostic

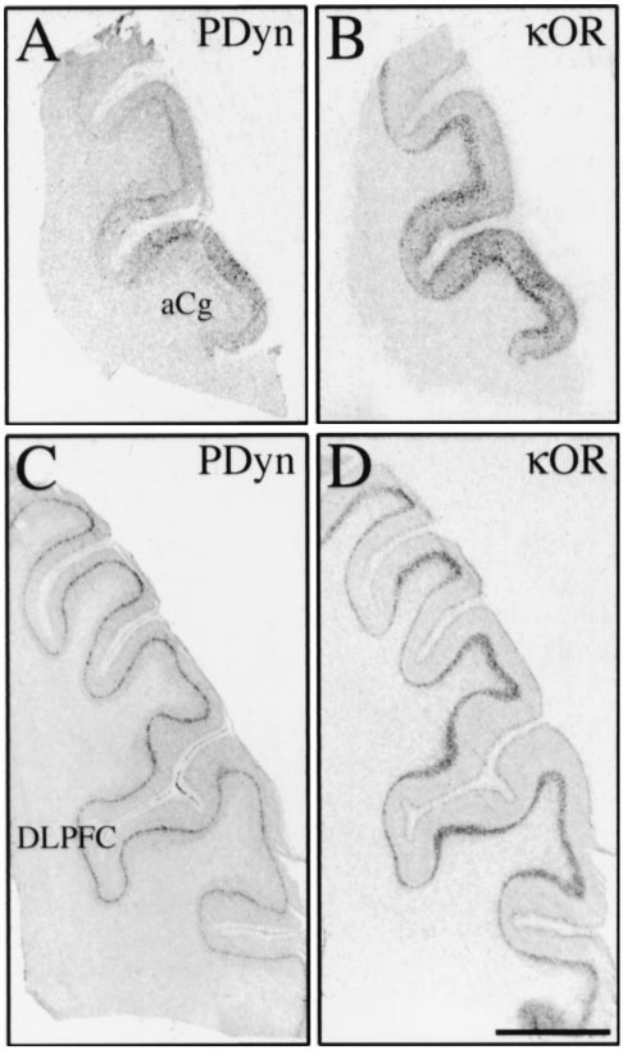

FIG. 1. Autoradiograms showing the expression pattern of the prodynorphin (PDyn; A,C) and kappa opioid receptor (κOR; B,D) mRNAs in the human anterior cingulate gyrus (aCg; A,B) and dorsolateral prefrontal cortex (DLPFC; C,D). Scale bar: 1 cm.

groups are presented in Table 1. No significant main effect of diagnosis was evident for the prodynorphin or the κ opioid receptor mRNA expression levels (Table 1; Figs. 2 and 3). A trend was, however, apparent for the κ opioid receptor mRNA expression levels in layer II of the prefrontal cortex [$F(3,50) = 2.2469$, $p = 0.0943$] due to the tendency of schizophrenic individuals to have lower (24.1%) expression levels than controls (Fig. 3B). A trend was also evident for the prodynorphin mRNA expression in the mean of all layers in the prefrontal cortex [$F(3,50) = 2.3883$, $p = 0.0799$]. Though the mRNA expression levels varied considerably between the subjects (the normal control group generally showed the least variability), the same cortical laminar pattern was evident in all subjects for both opioid markers. Five subjects (three major depressives, two bipolar) consistently showed extremely low expression levels of the mRNAs studied in both the prefrontal and cingulate cortices, but there was no direct association with any demographic characteristic, drug use history, or available toxicological information (e.g., antidepressant medication).

Neither age, gender, hemisphere side, nor suicide as a cause of death had any effect on the prodynorphin or κ opioid receptor mRNA expression levels in the cortex. In addition, the onset and duration of disease did not significantly influence the dpm/mg values measured in the cingulate and prefrontal cortices of the psychiatric groups. The lifetime antipsychotic treatment (fluphenazine) also was not associated with the opioid mRNA expression levels in the schizophrenic and bipolar disorder subjects.

Multivariate analysis revealed a significant main effect for the history of marihuana use for the prodynorphin mRNA levels measured in the cingulate cortex [$F(2,50) = 3.2337$, $p = 0.0478$]. *Post-hoc* analysis revealed that subjects with a history of past, but not recent, marihuana use had significantly higher (64 %) prodynorphin mRNA expression levels compared to those individuals with no drug use ($p < 0.05$, Tukey-Kramer). In addition, prodynorphin mRNA expression levels in the prefrontal cortex were found to be significantly enhanced [29–53%; mean all layers, $F(1,50) = 4.8706$, $p = 0.0319$; layer IV, $F(1,48) = 4.8210$, $p = 0.0330$; layer II, $F(1,50) = 4.2259$, $p = 0.0451$] in subjects with a recent history of stimulant use (only two categories available for analysis: no use or recent use).

DISCUSSION

This is the first report regarding prodynorphin and κ opioid receptor mRNA expression levels in the prefrontal and cingulate cortices of subjects with specific psychiatric diagnoses. We confirm the characteristic cortical lamination expression patterns of both mRNAs that has been described previously [21,37]. However, no significant effects of diagnosis on the prodynorphin or κ opioid receptor mRNA levels were detected in either cortical region and none of the psychiatric groups showed evidence for a substantial deviation from the lamination patterns found in the control brains. We did detect a diagnosis group trend in the mRNA expression levels of the κ opioid receptor and prodynorphin in the prefrontal cortex, but these findings did not reach statistical significance. The ability to observe significant effects of diagnosis on the cortical prodynorphin/κ opioid receptor genes could have been obscured by the sensitivity of these mRNAs to demographic differences between the subjects and/or postmortem tissue handling. There was, however, no main effect of factors such as age, gender, hemisphere side, suicide, or history of alcohol use, but PMI did influence both mRNAs in the cortical specimens. In view of the variability in the expression of both mRNAs found in the diagnostic groups, PMI and drug use history, although included in the statistical analysis, may contribute in part to the variability and blur possible underlying disease effects. The PMI impact on the prodynorphin and κ opioid receptor mRNA levels was not striking, but it is still reasonable that future mRNA studies of the cortical prodynorphin/κ opioid receptor system limit the PMI criteria to <30 h (a time period that did not significantly influence the prodynorphin and κ opioid receptor cortical mRNA expression levels; unpublished data). The high comorbidity between substance abuse and psychiatric disorders [40], however, makes it virtually impossible to study individuals diagnosed with schizophrenia or affective disorders who have not (ab)used addictive substances at some time in their lives.

Although no significant diagnosis-related effects on the prodynorphin nor κ opioid receptor mRNAs were found in the prefrontal and cingulate cortices, a role for the cortical dynorphin/κ opioid receptor system in these mental disorders cannot completely be discounted. Future studies should be carried out at the microscopic level to assess whether there are specific subpopulations of prodynorphin and/or κ opioid receptor mRNA-expressing cortical cells that are selectively impaired in the psychiatric subjects. Moreover, it is still conceivable that modifications in the post-transcriptional phase of the mRNA life

TABLE 1

PRODYNORPHIN (PDyn) AND κ OPIOID RECEPTOR (κOR) mRNA EXPRESSION LEVELS (MEAN ± SEM EXPRESSED IN dpm/mg) IN DIFFERENT LAYERS OF THE ANTERIOR CINGULATE AND DORSOLATERAL PREFRONTAL CORTICES IN NORMAL CONTROLS AND SUBJECTS WITH A PSYCHIATRIC DIAGNOSIS

	Control	Major Depression	Bipolar Disorder	Schizophrenia
Cingulate cortex				
PDyn mean of all layers	10.3 ± 0.9	7.1 ± 1.1	10.4 ± 1.7	10.1 ± 1.3
κOR mean of all layers	65.0 ± 3.4	57.4 ± 5.2	67.7 ± 7.2	71.1 ± 6.8
κOR layer II	89.5 ± 4.9	69.7 ± 6.0	89.5 ± 4.9	81.5 ± 5.3
κOR layer III	53.4 ± 4.1	49.1 ± 4.5	59.9 ± 7.2	59.5 ± 7.0
κOR layer V/VI	102.7 ± 5.4	95.1 ± 9.5	106.7 ± 9.6	111.8 ± 7.6
Prefrontal cortex				
PDyn mean of all layers	27.6 ± 2.6	18.7 ± 3.6	19.3 ± 2.9	20.6 ± 2.8
PDyn layer II–III	20.1 ± 2.6	13.6 ± 3.0	14.3 ± 2.9	15.5 ± 2.6
PDyn layer IV	63.0 ± 7.6	48.2 ± 7.3	52.1 ± 8.1	53.6 ± 4.9
κOR mean of all layers	26.9 ± 5.6	24.9 ± 2.2	25.1 ± 2.7	25.1 ± 1.4
κOR layer II	31.1 ± 1.7	26.9 ± 2.6	25.2 ± 2.7	23.6 ± 1.5
κOR layer III–IV	13.3 ± 1.2	12.2 ± 1.4	11.7 ± 1.3	10.7 ± 1.0
κOR layer V/VI	88.1 ± 4.3	83.4 ± 7.1	82.4 ± 8.1	91.1 ± 4.7

cycle or of their translated proteins influence, e.g., ligand binding properties and turnover. Finally, impairment of the prodynorphin/κ opioid receptor system in association with affective disorders might be more prominent in other cortical regions, such as the allocortex. Recently, a strong reduction of the prodynorphin mRNA expression was observed in the amygdaloid complex of subjects diagnosed with affective disorder [22].

As mentioned above, the history of drug use appeared to influence the expression levels of both opioid mRNAs. It has been repeatedly observed that the use of cocaine upregulates the prodynorphin mRNA expression in the striatum of rats [12,23,50] and humans [25]. In addition, a few studies have shown an upregulation of the κ opioid receptor mRNA in the rat [55] and human brain [51] following cocaine. These findings are supported in part by the current observations that there is a significant association between the recent history of stimulant use and increased prodynorphin mRNA expression in the prefrontal cortex. Moreover, the results showed a significant alteration of the prodynorphin mRNA expression in association with the history of marihuana use. Experimental animal studies have reported that activation of the cannabinoid receptor has a dose dependant influence on dynorphinergic neurons in the rat [31] and that cannabinoid tolerant mice display cross-tolerance to κ agonists [58]. Our data suggests that may also exist an association of marihuana with the endogenous κ opioid receptor system in humans and that this effect may be long-lasting. The apparent association between stimulant and

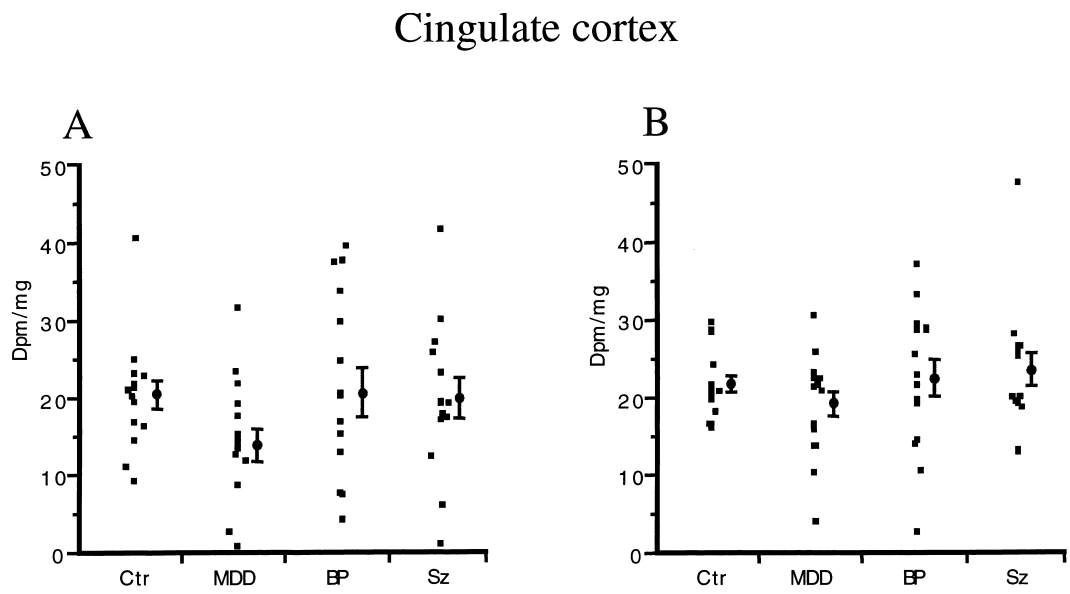

FIG. 2. Scatterplots showing examples of prodynorphin (A) and κ opioid receptor (B) mRNA expression levels (expressed as dpm/mg; mean ± SEM) in the cingulate cortex (mean all layers) of normal controls (Ctr) and subjects diagnosed with major depression (MDD), bipolar disorder (BP), or schizophrenia (Sz). No significant group differences were detected.

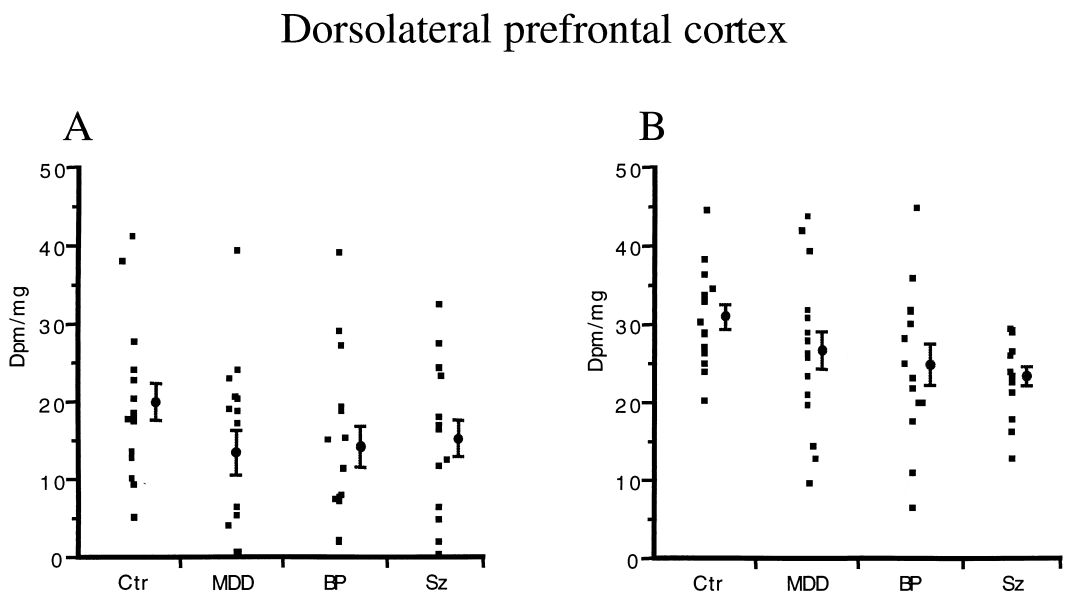

FIG. 3. Scatterplots showing examples of prodynorphin (A) and κ opioid receptor (B) mRNA expression levels (expressed as dpm/mg; mean ± SEM) in the dorsolateral prefrontal cortex (layer II) of normal controls (Ctr) and subjects diagnosed with major depression (MDD), bipolar disorder (BP), or schizophrenia (Sz). No significant group differences were detected.

marihuana use on the prodynorphin and κ opioid receptor mRNA expression levels in these cortical regions has to be confirmed in a brain collection focused on drug abuse and which includes more detailed toxicological analyses.

In conclusion, the current data do not support a strong role for altered prodynorphin or κ opioid receptor mRNA levels in the prefrontal and cingulate cortex in individuals diagnosed with schizophrenics, bipolar disorder or major depression.

ACKNOWLEDGEMENTS

This work was funded by the Karolinska Institute, Swedish Medical Research Council (11252), and the Stanley Foundation. Postmortem brains were donated by the Stanley Foundation Brain Consortium courtesy of Drs. Llewellyn B. Bigelow, Juraj Cervenak, Mary M. Herman, Thomas M. Hyde, Joel E. Kleinman, José D. Paltán, Robert M. Post, E. Fuller Torrey, Maree J. Webster, and Robert H. Yolken. We thank Mrs. Barbro Berthelsson and Miss Pia Eriksson for their valuable technical assistance and Elisabeth Berg (Karolinska Institute Statistical Department) for helpful statistical support.

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