5-1-2008

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Citation Information

Xiang, Lianbin; Szebeni, Katalin; Szebeni, Attila; Klimek, Violetta; Stockmeier, Craig A.; Karolewicz, Beata; Kalbfleisch, John; and Ordway, Gregory A.. 2008. Dopamine Receptor Gene Expression in Human Amygdaloid Nuclei: Elevated D4 Receptor mRNA in Major Depression. *Brain Research*. Vol.1207 214-224. https://doi.org/10.1016/j.brainres.2008.02.009 ISSN: 0006-8993

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Published in final edited form as:
Brain Res. 2008 May 1; 1207: 214–224. doi:10.1016/j.brainres.2008.02.009.

Dopamine Receptor Gene Expression in Human Amygdaloid Nuclei: Elevated D4 Receptor mRNAs in Major Depression

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Abstract

Previous findings from this laboratory demonstrating changes in dopamine (DA) transporter and D2 receptors in the amygdaloid complex of subjects with major depression indicate that disruption of dopamine neurotransmission to the amygdala may contribute to behavioral symptoms associated with depression. Quantitative real-time RT-PCR was used to investigate the regional distribution of gene expression of DA receptors in the human amygdala. In addition, relative levels of mRNA of DA receptors in the basal amygdaloid nucleus were measured postmortem in subjects with major depression and normal control subjects. All five subtypes of DA receptor mRNA were detected in all amygdaloid subnuclei, although D1, D2, and D4 receptor mRNAs were more abundant than D3 and D5 mRNAs by an order of magnitude. The highest level of D1 mRNA was found in the central nucleus, whereas D2 mRNA was the most abundant in the basal nucleus. Levels of D4 mRNA were highest in the basal and central nuclei. In the basal nucleus, amounts of D4, but not D1 or D2, mRNAs were significantly higher in subjects with major depression and depressed suicide victims, as compared to control subjects. These findings demonstrate that the D1, D2 and D4 receptors are the major subtypes of DA receptors in the human amygdala. Elevated DA receptor gene expression in depressive subjects further implicates altered dopaminergic transmission in the amygdala in depression.

Keywords

Dopamine receptor; gene expression; amygdala; depression; major depression; real-time PCR; human brain; dopamine

1. Introduction

The dopaminergic system plays an important role in the regulation of motor, cognitive, and emotional functions. Disturbances of the dopaminergic system have been strongly implicated

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in several neurological and psychiatric disorders, including Parkinson's disease, schizophrenia, and depressive disorders. Though most research on depressive disorders has focused on serotonin and norepinephrine, there is vast array of compelling clinical and laboratory animal evidence of a disruption of dopaminergic neuronal activity in depression (Dunlop and Nemeroff, 2007; Kapur and Mann, 1992; Roy et al., 1992; Swerdlow and Koob, 1987). For example, several drugs (neuroleptics, reserpine, α-methyl-*p*-tyrosine) that decrease dopaminergic transmission are known to precipitate depressive episodes (Charney, 1998; Willner, 1983a). In addition, low CSF concentrations of the dopamine (DA) metabolite, homovanillic acid (HVA; see (Willner, 1983b) and reduced venoarterial plasma concentrations of HVA have been reported in depression (Lambert et al., 2000). Consistent associations between DA receptor polymorphisms and depression have not been found (Dunlop and Nemeroff, 2007), although a recent metanalysis of 12 studies demonstrated a significant association of a specific repeat polymorphism of the D4 dopamine receptor (DRD4.2) with depression (López León et al., 2005). Results of neuroimaging studies of D2 receptor binding in subjects with major depressive disorder (MDD) have not been consistent and are reviewed by Dunlop and Nemeroff (2007). A recent functional magnetic resonance imaging study demonstrated that subjects with MDD have a greater behavioral response to the rewarding effects of amphetamine (Tremblay et al., 2005). In fact, the severity of depression correlates with the magnitude of reward experienced by administration of amphetamine, a drug that increases the synaptic availability of DA. This latter finding was used to argue that there is reduced synaptic DA in depression, leading to postsynaptic supersensitivity including supersensitivity to psychostimulants. The interpretation of postsynaptic supersensitivity is likely to be an oversimplification of the pathological process in depression, because numerous studies demonstrate that enhancement of D2-like receptor sensitivity is an important action of antidepressant drug action (Gershon et al., 2007). Despite our lack of a thorough understanding of DA pathobiology in depression, both preclinical and clinical findings suggest that brain dopaminergic activity is reduced in depression.

A region of the brain that is richly innervated by DA and that has considerable relevance to depression is the amygdala. The amygdala is involved in many emotion-laden behaviors and stress-related responses associated with depression (Ramel et al., 2007; Whalen et al., 2002). In fact, functional abnormalities in the amygdala correlate with the severity of MDD, i.e. resting cerebral blood flow and metabolism correlate positively with ratings of depression severity. In addition, antidepressant treatment reduces amygdala metabolism towards normal in MDD patients (Drevets, 2003). Patients with MDD show a greater activation of the amygdala in response to sad facial expressions, an effect that is attenuated by antidepressant treatment (Fu et al., 2004; Surguladze et al., 2005). Abnormal amygdala volume has also been demonstrated in MDD subjects, but consistent changes have not been observed across studies (Drevets, 2003), possibly because of unexpected familial and perhaps genetic influences on amygdala size (Munn et al., 2007). Low glial cell density has been observed in postmortem amygdala from MDD subjects (Bowley et al., 2002; Hamidi et al., 2004), as has been observed in the prefrontal cortex (Rajkowska et al., 1999), suggesting that reduced glia support to neuronal functions contribute to amygdala pathology in depression. Because of the important role of the amygdala in emotion processing and the putative role of DA in depression pathology, we previously investigated DA indices in postmortem amygdala from subjects with MDD. We found an elevation of D2 DA receptors and a reduction of DA transporters in the amygdala of subjects with MDD as compared to psychiatrically normal control subjects (Klimek et al., 2002). Remy and coworkers (2005) observed lower [^{11}C]RTI-32 binding (an *in vivo* marker of DA and norepinephrine transporters) in the amygdala in depressed Parkinson's patients relative to non-depressed Parkinson's patients, also implicating an association of a reduced number of DA transporters (or reduced DA innervation) in the amygdala with depression. Interestingly, administration of a DA neurotoxin that produces DA depletion results in upregulation of D2 DA receptor s (Angulo et al., 1991) and a reduction in DA transporters in

the rat brain (Gordon et al., 1996). Hence, postmortem receptor findings in MDD subjects suggest that amygdala pathology in depression may be associated with deficient DA transmission in the amygdala.

Five distinct subtypes of DA receptors mediate the actions of DA. D1 and D5 receptors belong to the D1 subfamily, and D2, D3, and D4 represent the D2 subfamily; each receptor subtype having a distinct pharmacological profile. DA receptors have been extensively characterized with regard to the relative distributions of receptor proteins and mRNAs in a number of regions of the brain (Hall et al., 1996; Kessler et al., 1993; Meador-Woodruff et al., 1996; Smiley et al., 1994). However, few studies have concentrated on gene expression of DA receptors in the subregions of the human amygdala, and gene expression levels of amygdala DA receptors in depression have not been investigated. The present study aimed to clarify the quantitative distribution of mRNA of the five subtypes of DA receptors in subnuclei of the human amygdala. The present study also sought to determine whether D2 receptor upregulation in depression observed previously (Klimek et al., 2002) may be related to an increase in D2 receptor gene expression. In previous work, D2 receptor binding was highest in the basal amygdaloid nucleus, a region where an elevation of D2 receptor binding and a decrease in DAT binding was observed in MDD subjects as compared to normal control subjects. Hence, the basal nucleus was the focus of the present investigation of DA receptor gene expression changes in depression. The basal nucleus is part of the basolateral complex and is classically considered a place of formation of conditioned and unconditioned stimulus associations in fear conditioning, and plays a major role in regulating memory consolidation, particularly emotionally charged memories (McIntyre et al., 2003). The basal nucleus also has reciprocal connections with medial and orbital frontal cortex, areas where reduced activity has been observed in MDD (Drevets, 2007). DA is known to enhance memory retention in the basolateral amygdala (Lalumiere et al., 2004; LaLumiere et al., 2005) and putative altered dopaminergic input to the amygdala could contribute to the link between disrupted processing of emotionally charged stimuli and increased amygdaloid neural response that has been observed in MDD subjects (Surguladze et al., 2005). The basal nucleus also communicates directly with the major output nucleus of the amygdala, the central nucleus (Knapska et al., 2007)), which projects to brainstem nuclei such as the locus coeruleus also known to be dysregulated in MDD (Ordway, 2007).

2. Results

DA receptor mRNA distribution

Initial experiments determined the quantitative distribution of the 5 subtypes (D1–D5) of DA receptor mRNA in five amygdaloid subnuclei (Figure 1), using tissue from seven psychiatrically normal control subjects. All PCR reactions were validated for the specificity of primers by melting curve analysis and agarose gel electrophoresis. DNA fragments with the expected size are shown in agarose gel electrophoresis of PCR reactions for the five DA receptors. In order to obtain copy numbers of the 5 DA receptor mRNAs, standard curves for each subtype were established using the corresponding plasmid cDNAs (Figure 2).

D1, D2, and D4 mRNAs were expressed in the amygdala at markedly higher levels than were D3 and D5 mRNAs (Figure 3), with the high expression genes (D1, D2, D4) differing in concentration by approximately an order of magnitude from the low expression genes (D3 and D5). Statistically significant differences in quantitative distributions of D1 $F(4,30)=8.03$; $p<0.0005$), D2 ($F(4,30)=21.7$; $p<0.0001$), and D4 $F(4,30)=3.63$; $p<0.05$) receptor gene expressions amongst the amygdaloid nuclei were observed. However, for the low expression genes, D3 and D5, no significant difference in expression levels were observed among the nuclei.

The highest concentrations of D1 receptor mRNA were found in the central, lateral and basal amygdaloid nuclei, where its concentrations were 4- to 7-fold higher than that in the accessory basal and cortical nuclei. In contrast to D1 receptor gene expression, the expression of the D2 receptor gene was most abundant in basal amygdaloid nucleus, where its expression was approximately 4- to 20-fold higher than that in the other amygdaloid nuclei. D2 receptor mRNA was least abundant in the lateral nucleus, with intermediate levels of expression in the accessory basal, cortical and central nuclei. D4 receptor gene expression was more evenly distributed across the amygdaloid nuclei than were D1 and D2 receptor mRNA, although the basal and central nuclei had the highest level of D4 gene expression.

DA receptor mRNAs in major depression

ΔDC_T values for D1 and D2 receptor gene expressions did not significantly differ comparing the normal control and MDD subject groups. The mean ΔC_T value for D4 receptor mRNAs in the basal nucleus of the amygdala from MDD subjects was significantly lower than that from normal control subjects ($p<0.03$; Figure 4), indicating higher D4 mRNA levels in the MDD group. Conversion of ΔC_T values to fold changes ($2^{-\Delta\Delta CT}$; Livak and Schmittgen, 2001) showed that D4 receptor gene expression was approximately 2-fold higher, respectively, in MDD subjects as compared to control subjects. Although the common feature of all subjects in the psychiatric group was MDD, two subjects with MDD did not die as a result of suicide. The average ΔC_T for D4 receptor gene expression of the two MDD subjects that did not die by suicide was 2.5 cycles lower than the average of their two matched control subjects, demonstrating that these two subjects had elevations in D4 mRNA very similar to the elevations in the MDD subjects that died as a result of suicide.

Postmortem tissue variables, mRNA quality and quantitation

The possibility that variables unrelated to psychiatric status contributed to differences in gene expression between depressive and control subjects was extensively considered. Study groups were carefully matched for several parameters to reduce the influence of potentially confounding issues. However, exact matching of all variables is not possible in postmortem studies given the number of variables and the limitation of the availability of brain tissue from suitable subjects. Postmortem intervals, pH values, and RIN values appear in Tables 1 (control subjects) and Table 2 (depressed subjects). A summary of the averages of these values, including ages, appears in Table 3. There were no statistically significant differences between the 3 study groups comparing ages, PMIs, pH values, or RIN values.

Others have demonstrated an association between pH and RNA quality [34–36], drawing our particular attention to these variables. No significant correlation between pH and RIN was observed, even when all data were pooled from both study groups ($r^2 = 0.14$; $p > 0.05$; Figure 5). This is likely related to the fact none of the brain tissues had pH values below 6.2. In our experience, tissues with pH values below 6.0 often, but not always, have low quality RNA. In addition, no correlations between PMI and RIN, or PMI and pH were observed. As expected, RIN values were highly correlated with 18S/28S RNA ratios ($r^2 = 0.53$, $p < 0.0001$; data not shown), another related index of RNA integrity. Neither age, pH, PMI, nor RIN significantly correlated with ▵Ct values for D1, D2 or D4 receptor gene expressions in either study group.

Finally, there were 3 subjects in the control group and 4 subjects in the MDD group that were smokers at the time of death. Given the known effects of nicotine on DA neurotransmission, we evaluated the possible association of the diagnosis of nicotine dependence on DA receptor gene expression. Since there was a small number of smokers in both groups, we combined control and MDD smokers and compared them to the combined control and MDD non-smokers. There were no significant differences between these two groups for all 3 receptor mRNAs (ΔCt values reported; D1: smokers 6.85 ± 0.29, non-smokers 7.52 ± 0.33; D2: smokers

6.93 ± 0.28, non-smokers 6.85 ± 0.17; D4: smokers 7.82 ± 0.25, non-smokers 7.59 ± 0.29), albeit the comparison of smokers to non-smokers in the MDD group is complicated by the fact that there is a significant difference in D4 gene expression between controls and MDD subjects. Nevertheless, the data do not support a relationship between nicotine dependence and DA receptor gene expression in the basal amygdala.

3. Discussion

This study details for the first time the quantitative expression pattern of mRNAs for the 5 DA receptors in the human amygdala subnuclei, using quantitative real-time RT-PCR. We confirm previous identifications of DA receptor gene expression in the amygdala, demonstrate the predominance of D1, D2 and D4 mRNA in the amygdala, and clarify the relative abundance of the different receptor mRNAs in the various amygdaloid subnuclei. In addition, we report elevated levels of D4 DA receptor mRNA in the amygdala from subjects with MDD as compared to psychiatrically normal control subjects.

All 5 DA receptors are widely present in the brain including in the limbic system (Meador-Woodruff et al., 1994). However, few previous studies have investigated the expression of these receptors in the amygdaloid complex. Low levels of expression of the D1 receptor gene have been found in basal and lateral amygdala using *in situ* hybridization (Hurd et al., 2001). In the present study, both D1 and D5 mRNAs were observed in the amygdala, although D1 gene expression was much more robust. D5 receptor gene expression has been demonstrated in several brain regions (Bouthenet et al., 1991; Choi et al., 1995; Huntley et al., 1992; Rappaport et al., 1993), but has not been previously reported in the human amygdala. We found D5 receptor mRNA to be expressed at low levels in all amygdaloid nuclei relative to D1 receptor mRNA. The low level of expression suggests that the D5 receptor may play only a minor role in mediating DA signaling in the amygdala, although it is possible that this receptor may be expressed abundantly on a small number of neurons and play a major role in DA signaling on those neurons. This ambiguity is an inherent limitation of the method used to quantify mRNAs in the present study.

Messenger RNAs for the D2-like receptors are expressed widely in the human brain (Bouthenet et al., 1991; Jackson and Westlind-Danielsson, 1994), but only the D2 and D3 receptor mRNAs have been previously reported in the human amygdala. In agreement with previous reports (Hurd et al., 2001; Joyce et al., 1991), we found that D2 receptor mRNA was abundant in the basal amygdala and expressed at lower levels in the lateral nucleus. D2 mRNA also was found in the other subregions of amygdala including basal accessory, cortical, and central areas at lower levels compared to the basal nucleus. Others have reported low levels of D3 receptor mRNA in the human amygdala (Gurevich and Joyce, 1999; Murray et al., 1994). Gurevich and Joyce (1999) found no detectable signal for the D3 receptor mRNA in the central nucleus, using in situ hybridization. We found D3 receptor mRNA to be present in all amygdaloid nuclei, including the central nucleus, albeit in relatively low concentrations in all regions. We used RT-PCR to evaluate expression levels, a method capable of detecting very low expression levels. Thus, the different techniques used to measure mRNA likely contributed to the discrepancy in findings regarding D3 receptor mRNA between our study and that of Gurevich and Joyce (1999). Similar to the D5 receptor, the level of D3 mRNA was considerably lower than that of the D1, D2 and D4 receptor mRNAs.

D4 receptor mRNA has been observed in several brain regions (O'Malley et al., 1992; Van Tol et al., 1991), but there is no previous description of D4 receptor mRNA in the human amygdala. The present findings demonstrate robust expression of the D4 receptor gene throughout the human amygdaloid nuclei, with levels of expression similar to the D1 and D2 receptor genes. The level of D4 gene expression indicates that this receptor may play a significant role in

transducing dopaminergic signaling in the amygdala. Most research investigating the D2 DA receptor involvement in behaviors associated with the amygdala have used antagonists that have high affinities for D2 and D3 receptors. Research designed to investigate the role of D4 DA receptor-mediated signaling in the amygdala is needed.

Using single photon emission tomography and [^{123}I]iodobenzamide, an increase in D2 receptor density in the striatum in depressed subjects has been reported (D'Haenen and Bossuyt, 1994; Shah et al., 1997), although no change in *in vivo* D2 receptor binding has also been observed in depression (Parsey et al., 2001). We previously (Klimek et al., 2002) demonstrated elevated [^{125}I]epidepride binding to D2 receptors, reduced [^{125}I]RTI binding to DA transporter, and unchanged [^{3}H]SCH 23390 binding to D1 receptors in amygdaloid subnuclei (including the basal nucleus) from MDD subjects as compared to psychiatrically normal control subjects. In these receptor studies, the specific D2-like subtype (D2, D3 or D4) of DA receptor that was labeled by [^{125}I]epidepride or the chemically related [^{123}I]iodobenzamide was not certain. The affinities of epidepride for D2 vs D4 receptors has not been published. In the present study, D2 and D4 receptor mRNAs were found to be the predominant D2-like receptor mRNAs in the amygdala, and D4 mRNA was significantly elevated in depressed subjects. Based on the present study, translated D4 mRNAs (to receptor proteins) may have contributed to the elevated binding of [^{125}I]epidepride to D2-like receptors in the basal nucleus of the amygdala reported previously (Klimek et al., 2002). No changes in D1 gene expression in the amygdala reported here corroborate previous findings of no differences in D1 receptor binding in the amygdala in depressive subjects (Klimek et al., 2002).

A large body of research implicates a deficiency of DA in the pathophysiology of depression (Dunlop and Nemeroff, 2007), as well as a role of D2 receptors in the neurochemical disruption. Drugs that block DA receptors or deplete DA can induce depressive symptoms (Berman et al., 1999; Willner, 1983a; Willner, 1983b). Also, Parkinson's disease (low brain DA) is associated with a high incidence of depression (Leentjens et al., 2003; Lemke et al., 2004; Nuti et al., 2004). DA agonists alleviate movement disorders but also elevate mood in depressed Parkinson's subjects (Lemke et al., 2005). In rats, acute stress increases DA release (Finlay et al., 1995; Inglis and Moghaddam, 1999), while exposure to repeated stress reduces brain DA concentrations, such as in the nucleus accumbens where sustained DA reductions are temporally correlated with behavioral deficits modeling depression (Gambarana et al., 1999; Mangiavacchi et al., 2001). Repeated antidepressant treatment normalizes DA deficits produced by chronic stress (Gambarana et al., 1999). Exposure of rats to chronic social stress results in increased D2, but not D1, receptor binding in specific brain regions (Lucas et al., 2004) and imipramine treatment reverses chronic mild stress–induced anhedonia and simultaneously reverses decreased D2, but not D1, receptor binding in the limbic forebrain that is associated with decreases in the performance of rewarded behavior (Papp et al., 1994). Besides chronic stress, pharmacological reductions of dopaminergic transmission (haloperidol treatment, 6-hydroxydopamine lesions of mesencephalic dopaminergic neurons, or reserpine treatment) results in elevations of D2 receptor protein and gene expression in the striatum and nucleus accumbens (Angulo et al., 1991; Papp et al., 1994). Together, these laboratory animal and clinical data urge the interpretation that elevated D2-like receptor binding (D'Haenen and Bossuyt, 1994; Klimek et al., 2002; Shah et al., 1997) and gene expression (present study) reflect deficient limbic dopaminergic transmission in depression. Alternative hypotheses could also explain the present data. For example, elevated D4 gene expression may be pre-existing in MDD subjects, e.g. as a result of polymorphic differences, rather than as a result of compensation for reduced DA. The D4 DA receptor gene (DRD4) is polymorphic and one polymorphism, a 48-base pair repeat that can have functional consequences, has received much attention in psychiatry. The 3 most common repeat polymorphisms are DRD4.2 (2 repeats), DRD4.4 and DRD4.7. DRD4.2 and DRD4.4 are associated with higher gene expression than DRD4.7 (Schoots and Van Tol, 2003). A recent metanalysis demonstrated that the DRD4.2

allele is a risk allele for depression symptommatology (Lopez Leon et al., 2005). Hence, an increased prevalence of DRD4.2 (higher expression levels) in our MDD group, could have resulted in fewer DRD4.7 subjects (lower expression levels) in this group, and could have contributed to the observed increase in D4 mRNA in MDD.

Numerous in vivo studies demonstrate abnormalities of the amygdala in patients with MDD (Rosso et al., 2005; Siegle et al., 2007) (see (Drevets, 2003) for review, with exception (e.g. (Frodl et al., 2004)). Among neuronal circuits likely involved in the control of cognitive and emotional processes that are disrupted in depression, are projections from the amygdala and ventral tegmental area (dopaminergic) that converge in the medial prefrontal cortex, as well as prefrontal cortical projections to the amygdala. Recent imaging studies implicate reduced prefrontal cortical function that is linked to increased amygdala activity (Drevets, 1999; Siegle et al., 2007). Interestingly, animal studies demonstrate that dopaminergic mechanisms mediated by D1, D2, and D4 receptors, regulate the balance of excitatory and inhibitory transmission in the basolateral amygdala and medial prefrontal cortex (Floresco and Tse, 2007). Hence, depression-associated changes in expression of DA receptors may contribute to an imbalance of excitatory and inhibitory communication between the prefrontal cortex and amygdala.

There are a number of shortcomings with regard to this study that deserve attention. One issue not adequately addressed is whether DA alterations are associated with suicide behaviors that are separate from those associated with depression. Suicide was the method of death in 9 out of the 11 MDD subjects. The small number of subjects with MDD that died from natural causes precluded analysis of suicide vs natural death within the MDD group. The issue of the biology of suicide vs the biology of depression is one of the most difficult to investigate using postmortem tissues because the majority of available and acceptable tissues (including the lack of antidepressant drugs in toxicology) from depressed subjects who are depressed at the time of death, at least in our experience, are from those who committed suicide. Another issue is that 5 of the 11 MDD subjects had a history of antidepressant drug prescription. These drugs were not found in their blood or urine at autopsy suggesting that the subjects were not compliant with medication. Also, 4 of these 5 subjects committed suicide, which may have been contributed to by non-compliance, inadequate dosing, and/or treatment refractoriness. Effects of repeated antidepressant treatment on DA receptor mRNAs in the rat brain have been reported, but effects are variable (increase, decrease or no change) depending on the type of antidepressant, the receptor subtype, and the region of brain studied (Ainsworth et al., 1998; Dziedzicka-Wasylewska et al., 1997; Dziedzicka-Wasylewska et al., 2002; Huzarska et al., 2006; Kameda et al., 2000; Lammers et al., 2000). To our knowledge, DA receptor gene expression in the amygdala following antidepressant drug treatment to rats has not been studied. The small number of subjects with prescription histories precludes separate evaluation of the MDD subjects having antidepressant prescription histories. It is worth noting that the ΔCt values for D4 mRNA of these 5 MDD subjects ranged from highest to the lowest among all subjects with MDD, pointing to no obvious relationship between prescription history and DA gene expression levels.

The present findings demonstrate that the predominant DA receptor genes expressed in the amygdala are the D1, D2, and D4 subtypes. Levels of gene expression of the D4 receptor are elevated in the basal amygdaloid nucleus of depressed subjects relative to normal control subjects. These latter findings, along with converging results from laboratory animal studies, other human postmortem findings from depressed subjects, *in vivo* imaging studies in depressed patients, and findings regarding the association of depression with Parkinson's disease, provide strong justification for further investigation of the role of DA in the pathophysiology of depression.

4. Experimental Procedure

Human Subjects

Human brain tissue was collected at autopsy at the Cuyahoga County Coroner's Office in Cleveland, Ohio, in accordance with an approved Institutional Review Board Protocol, as described previously (Karolewicz et al., 2005). For the study of receptor mRNA distribution, discarded brain tissues from 7 subjects (5 males, 2 females) were collected at autopsy and detailed medication and psychiatric histories were unavailable. These subjects died as result of cardiovascular disease (4), gastrointestinal disorder (1), aspirin overdose (non-suicide) (1) and homicide (gun shot; 1). Based on autopsy records alone, there was no indication of psychiatric or neurologic history. Subjects had an average age of 48 ±5 y (average ± S.E.M.), an average postmortem interval of 22 ± 2 h, an average RNA integrity number (RIN; see below) of 7.0 ± 0.3, and an average brain tissue pH of 6.43 ± 0.07. Psychoactive drugs (oxycodone and ethanol) were detected in only one of these subjects.

For the study of relative gene expression levels in MDD, retrospective, informant-based psychiatric assessments were performed for all depressed and control subjects. The Structured Clinical Interview for DSM-IV Psychiatric Disorders (SCID-IV) or Schedule for Affective Disorders and Schizophrenia: lifetime version (SADS-L) was administered to next-of-kin as previously described (Stockmeier et al., 2002). Axis I psychopathologies were assessed and consensus diagnoses were reached in conference using information from the interview and medical records. Blood and urine samples from all subjects were examined by the coroner's office for psychotropic medications and substances of abuse. Psychiatric subjects available for study included many that had antidepressant and antipsychotic medication in their toxicology. However, only those subjects free of these drugs were used in the present study. Alcohol dependent subjects with MDD were also available, but these subjects were also not included in this study. Control subjects (2 females and 9 males) were individuals who acceded to natural causes of death (Table 1). All control subjects had no Axis I psychiatric diagnosis at the time of death and no history of an Axis I disorder, other than nicotine dependence, and no known history of substance abuse. Major depressive subjects studied differed from our previous study (Klimek et al., 2002) because of the lack of adequate amounts of tissue from subjects in the previous study. The MDD group consisted of 11 subjects (3 females and 8 males) with active MDD at the time of death, and who had died by natural deaths (2) and suicide (9) as listed in Table 2. Subjects in the MDD group had no known history of substance abuse except for subject KS32 who had a history of alcohol abuse, but was not alcohol dependent. Five of the 11 MDD subjects had a history of antidepressant prescriptions (fluoxetine, sertraline, nortriptyline, trazadone, buproprion) in medical records, but none of these subjects demonstrated positive toxicology for antidepressants at autopsy, indicating non-compliance to medication or termination of compliance prior to death. Other details of study subjects, not identified with specific subjects, appear in Table 3 and in *Results*. Details of individual subjects are not aligned in order to protect subject identities.

Dissection

Tissue blocks containing the amygdala were sectioned serially in the coronal plane beginning at their anterior border. Histological sections (20 μm) were collected at 2 mm intervals. Sections were processed for both Nissl staining and acetylcholinesterase (AChE) histochemistry (Sims and Williams, 1990) to permit the identification of specific amygdaloid nuclei and dissections of equivalent anatomical levels within these nuclei for all subjects (as performed in our previous study (Klimek et al., 2002)). Amygdaloid tissue from the different nuclei was collected at the point along its anterior-posterior extent where the basal nucleus is bifurcated (Mai et al., 1997; see Figure 1). The central nucleus was found in the angle between the basal and accessory basal nuclei and had no AChE reactivity. The lateral nucleus was easily distinguishable from

the other nuclei. AChE-stained slides were held over the tissue block containing the amygdala and a 25 gauge needle was used to mark the borders of the basal, accessory basal, and central nuclei. Between 2 histological sections (representing 2 mm of tissue along the anterior-posterior axis of the amygdala) at the point of bifurcation of the basal nucleus, 40 sections (50 μm) were collected and individual nuclei were dissected from each of the sections using a tissue punch, storing the punches at −80°C until assayed. The bore of the punch was 5 mm for the lateral nucleus, and 3.5 mm for all other nuclei.

RNA Preparation

Total RNA was extracted from tissue punches using RNAqueous Phenol-Free Total RNA Isolation Kit (Ambion, Austin, TX). RNA samples were quantitated using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Rockland, DE). The quality of RNA, computed as RIN (Schroeder et al., 2006) was assessed with an Agilent 2100e Bioanalyzer (Agilent Technologies, Santa Clara CA).

Recombinant plasmid DNA construction

cDNA fragments of DA receptor 1–5 (D1–D5) and the housekeeping genes β-actin and β2-microglobulin were generated by reverse transcription with SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA) and PCR using Platinum PCR SuperMix System (Invitrogen, Carlsbad, CA) with specific primer sets. Primers were designed to amplify regions that were common to all known splice variants (Table 4). PCR products were subcloned into the pCRII-TOPO TA cloning vector (Invitrogen, Carlsbad, CA). The sequence of each insert was confirmed by restriction mapping and DNA sequencing reaction (Research Core Facility, Dept. of Biochemistry, University of Mississippi Medical Center, Jackson, MS).

Real-time RT-PCR

Each sample of total RNA (0.5 μg) was DNase treated and reverse transcribed. RT minus controls were used to identify any contaminating genomic DNA. One microliter of the resulting cDNA was used as a template for real-time PCR using an iCycler (Bio-Rad, Hercules, CA) with SYBR Green (Stratagene, La Jolla, CA) Amplification was carried out using gene specific primers at a final concentration of 400 nM for the DA receptor and referernce genes. Each PCR amplification was performed in triplicate wells using the following temperature and cycling profile: 95°C for 10 min, 40 cycles of 95°C for 30 s, at the annealing temperature for 30 s (specified in Table 4), and 72°C for 1 min. The cycle threshold value (C_T) used to assess the quantity of target gene was determined as the point at which the increased fluorescence exceeded background fluorescence. For comparisons between control and MDD subjects, cDNAs from paired samples were amplified simultaneously on the same plate. In order to generate the standard curves to quantify each type of DA receptor mRNA in human amygdaloid subnuclei, known amounts of recombinant DNA (see above) were 5-fold serially diluted and then amplified. All samples cDNAs were measured in the range of the standard curve. The copy numbers of cDNA from each DA receptor mRNA were calculated based on the corresponding standard curve. Amplification efficiencies of primer sets ranged from 94% to 108%, except for D5 which was 125%. D5 was not used in the relative comparisons between control and MDD subjects, i.e. not compared relative to reference genes. D5 mRNA quantitation was estimated in the mRNA distribution study only using its own standard dilution curve.

Data analysis

The quantitative distribution of gene expression of DA receptors in the amygdala was analyzed using a one-way ANOVA. Post hoc comparisons between regions were made using the Newman-Keuls Multiple Comparison Test (GraphPad Prism version 4.00 for Windows;

GraphPad Software, San Diego, CA). For the analysis of expression data from depressed and control subjects, C_T values of DA receptor transcripts were normalized by subtracting the geometric mean (Vandesompele et al., 2002) of the C_T values of β-actin and β2-microglobulin gene expressions generated from the identical reverse transcribed mRNA samples. Resulting ΔC_T values provided relative gene expression levels for DA receptor transcripts between study groups. For calculation of fold-changes between control and MDD subjects, ΔC_T values were converted using the $2^{-\Delta\Delta CT}$ method (Vandesompele et al., 2002). Amplification efficiencies of target genes and the reference genes were evaluated using linear regression analysis. Data from major depressive and normal control subjects were statistically analyzed using an unpaired Student t-test. Data are presented as mean ± S.E.M.

Acknowledgements

The excellent assistance of the Cuyahoga County Coroner's Office, Cleveland, OH is greatly appreciated. This research was supported by the National Institute of Mental Health (MH63187, MH46692, MH02031, MH67996) and by the National Center for Research Resources (RR17701).

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Abbreviations

DA, dopamine; HVA, homovanillic acid; mRNA, messenger RNA; S.E.M., standard error of the mean; MDD, major depressive disorder; AChE, acetylcholinesterase; C_T, cycle threshold; RIN, RNA integrity number; CSF, cerebrospinal fluid; ANOVA, analysis of variance.

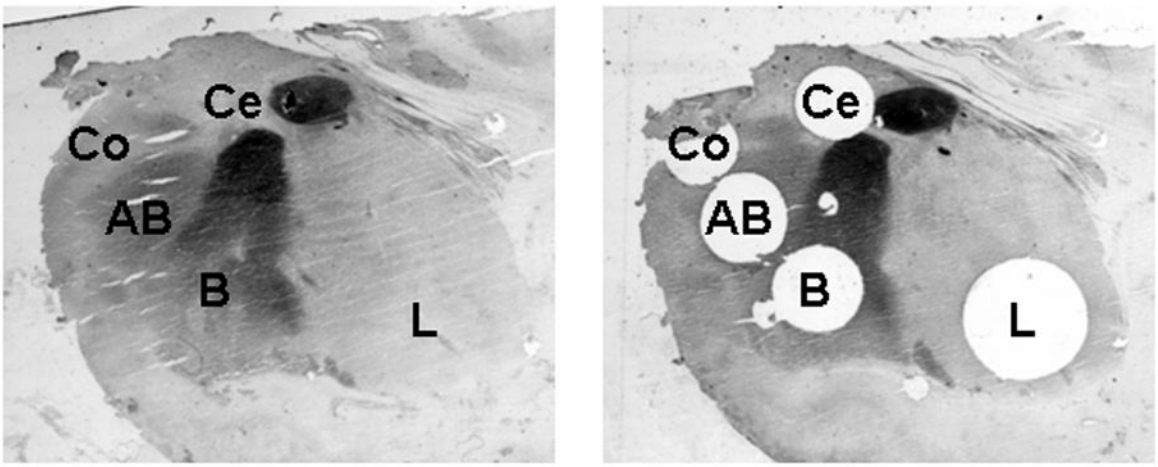

Figure 1.

Digital images of coronal sections of the human amygdala stained histochemically for acetylcholinesterase. The left panel shows a complete section stained for acetylcholinesterase, with amygdaloid nuclei labeled. The right panel illustrates a section stained for acetylcholinesterase, following the tissue punching of nuclei for RNA extraction. The cortical nucleus was close to the edge of the tissue block and a small piece of tissue on the upper side has shifted during the section mounting. Abbreviations of amygdaloid nuclei are B basal, L lateral, AB accessory basal, Co cortical, and Ce central.

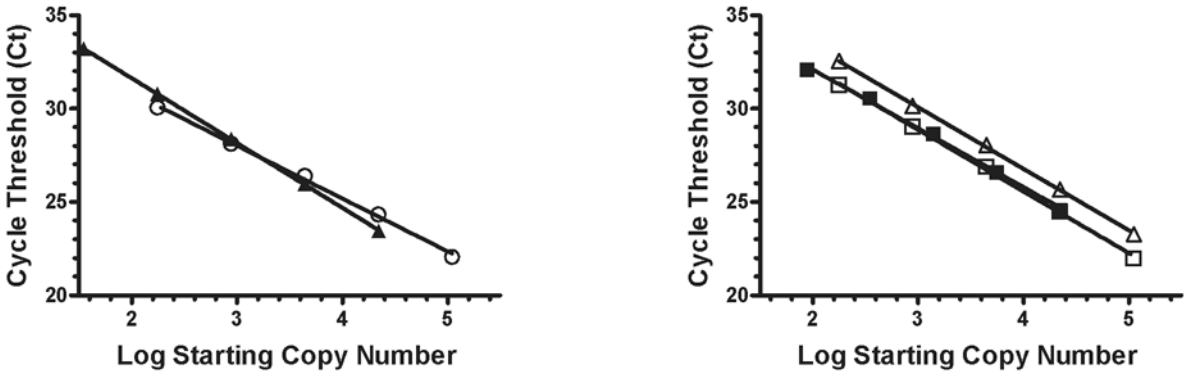

Figure 2.

The real-time RT-PCR sensitivity and linearity analysis of standards for cDNAs each of the five subtypes of DA receptor, derived from cloned cDNA plasmids. Standards for D_1-like (left panel; D_1 ▲, D_5 ○) and D_2-like (right panel; D_2 ■; D_3 ▵; D_4 □) receptor cDNAs were 5-fold serial dilutions of the cloned cDNA standards starting from 35 to 1.11×10^5 molecules per reaction amplified by the real-time PCR. The correlation coefficients (r^2) of the 5 standard curves were all greater than 0.99.

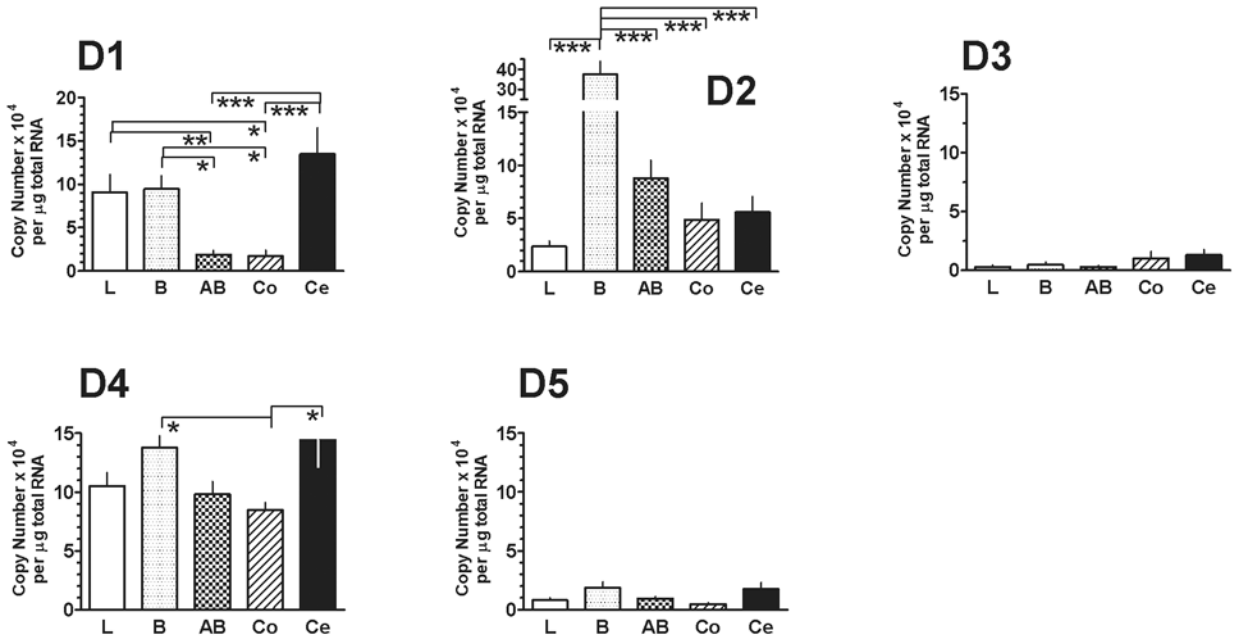

Figure 3.

Quantitative distribution of DA receptor gene expression in subregions of the human amygdala. Amounts of mRNA were quantified by real-time RT-PCR. The mRNA copy number for each DA receptor was calculated base on the standard curves shown in Figure 2. Abbreviations of amygdaloid nuclei are as described in Figure 1. Some lines depicting the standard error of the mean were drawn downward into the bars to aid in illustrating the distribution across all nuclei. Asterisks indicate statistical significance between bars of comparison denoted by connecting lines (* $P < 0.05$, ** $P < 0.01$, ***$P < 0.001$).

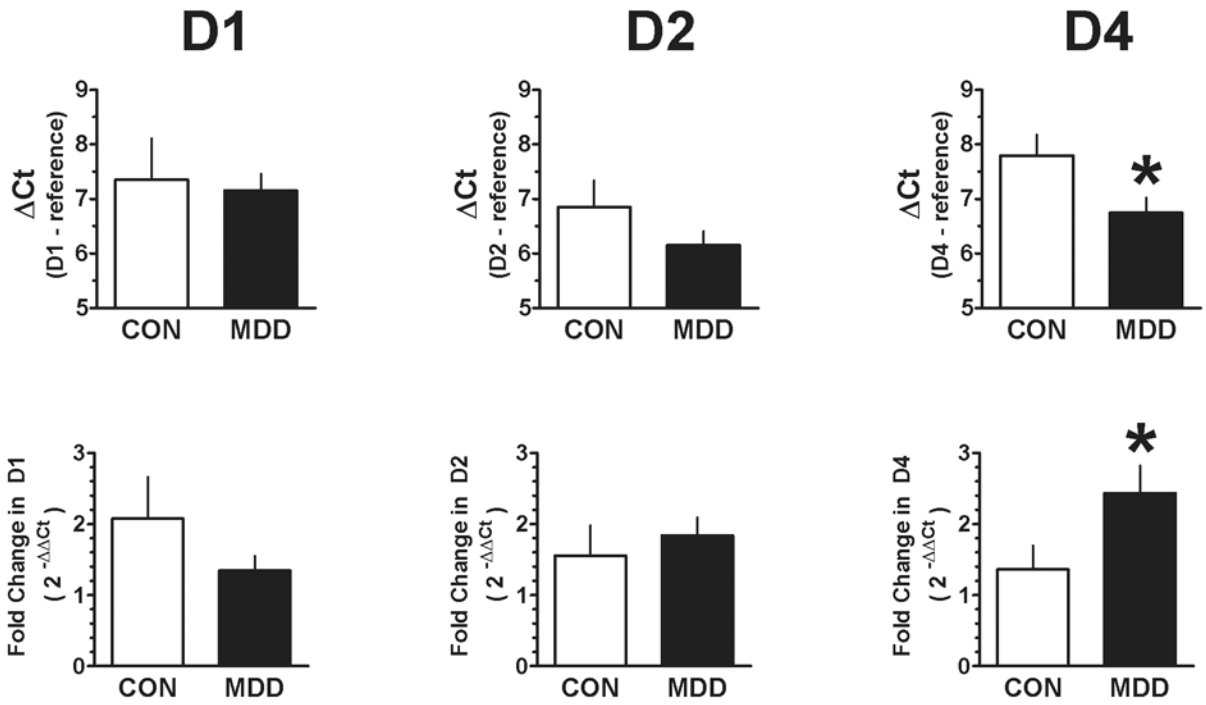

Figure 4.

Relative amounts of mRNA of DA receptors in the basal amygdaloid nucleus from psychiatrically normal control subjects (n=11) and subjects with MDD (n=11), expressed as either ΔCt (target gene minus reference genes; upper panels) or by fold change ($2^{-\Delta\Delta Ct}$; lower panels. Asterisks indicate statistically significant differences compared to control group (* $P < 0.05$).

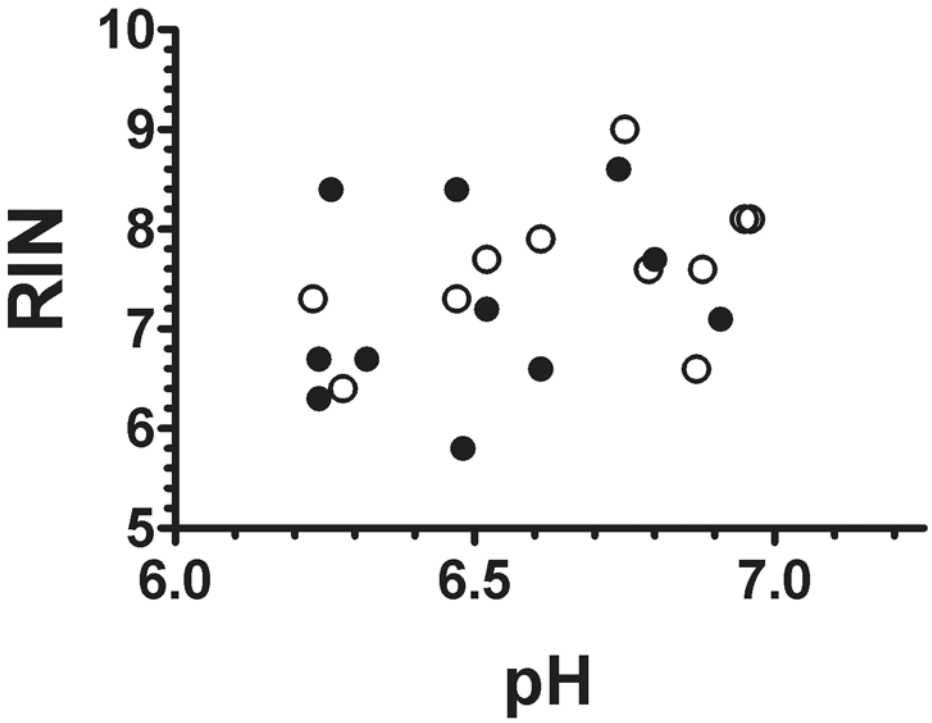

Figure 5.
Relationship between RNA integrity (RIN) and pH values in psychiatrically normal control (○) and MDD (●) subjects. RIN values were not significantly correlated with brain tissue pH.

Table 1

Demographic data of psychiatrically normal control subjects.

Subject	Brain pH	RIN[a]	PMI[b]	Toxicology	Cause of death	Psychiatric Diagnosis
RR	6.47	7.3	17	NDD[c]	acute hemorrhagic pancreat it is	no diagnosis
VV	6.52	7.7	19	lidocaine	heart disease	nicotine dependence
FF1	6.88	7.6	17	NDD	gun shot to chest	nicotine dependence
HH1	6.87	6.6	17	brompheniramine	heart disease	no diagnosis
BB1	6.28	6.4	17	NDD	heart disease	no diagnosis
KS31	6.79	7.6	6	lidocaine	heart disease	no diagnosis
KS39	6.61	7.9	24	ethanol	thrombophlebitis	no diagnosis[d]
KS41	6.95	8.1	19	NDD	heart disease	no diagnosis
KS43	6.96	8.1	24	NDD	heart disease	nicotine dependence
KS44	6.23	7.3	22	NDD	heart disease	no diagnosis
KS45	6.75	9.0	9	NDD	heart disease	no diagnosis[d]

[a] RIN, RNA integrity number

[b] PMI, postmortem interval

[c] NDD, no drugs detected

[d] smoking history unknown

Table 2

Demographic data of depressed subjects.

Subject	Brain pH	RIN[a]	PMI[b]	Toxicology	Cause of death	Psychiatric Diagnosis
TT	6.52	7.2	24	NDD[c]	suicide	major depression
WW	6.24	6.7	30	Codeine	suicide	major depression, nicotine dependence
GG1	6.91	7.1	18	Ethanol	suicide	major depression, nicotine dependence
JJ1	6.24	6.3	23	CO, phenobarbital, phenytoin	homicide	major depression
DD1	6.48	5.8	18	CO	suicide	major depression, dysthymic disorder
KS32	6.32	6.7	20	NDD	suicide	major depression, alcohol abuse, nicotine dependence
KS38	6.80	7.7	20	NDD	suicide	major depression[d]
KS40	6.26	8.4	17	NDD	CO poisoning	major depression
KS42	6.47	8.4	20	NDD	suicide	major depression, nicotine dependence
KS46	6.61	6.6	19	norpropoxyphene, propoxyphene	suicide	major depression, anxiety disorder NOS[d]
KS47	6.74	8.6	17	NDD	homicide	major depression[c]

[a] RIN, RNA integrity number

[b] PMI, postmortem interval

[c] NDD, no drugs detected

[d] smoking history unknown

Table 3

Summary of variables associated with study subjects

Study Group	n	Age	Postmortem Interval	pH	RIN[1]
Control subjects	11	49.4 ± 5.2	17.4 ± 1.7	6.66 ± 0.08	7.5 ± 0.2
MDD subjects	11	49.6 ± 4.5	20.6 ± 1.2	6.51 ± 0.07	7.2 ± 0.3

[1] RNA integrity number

Table 4

QPCR primers

Gene	Primer Sequence	Transcript Accession Number	PCR Product (#bp)	Annealing Temperature
DRD1	CTTAGGATGCTACAGACTTTGCCCTG CATGTGGGATCAGGTAAACCAGATTG	NM_000794	149	57°C
DRD2	TCTTCGGACTCAATAACGCAGACC GATGTAGACCAGCAGGGTGACAAT	NM_016574 NM_000795	119	58°C
DRD3	GAGGTGACAGGTGGAGTCTGGAATTTC GGCACAGAGATTAAGGATGCTGGCTG	NM_033663 NM_000796	101	60°C
DRD4	CTGTGCTGGACGCCCTTCTTC TTGAGGGCGCTGTTGACGTAG	NM_000797	118	60°C
DRD5	TGTCCATCCTCATCTCCTTCATTCC CTGGAGTCACAGTTCTCTGCATTCAC	NM_000798	159	60°C
ACTB	GCACCCAGCACAATGAAGATCAAG TCATACTCCTGCTTGCTGATCCAC	NM_001101	128	58°C
B2M	GTGCTCGCGCTACTCTCTCT TCTCTGCTGGATGACGTGAG	NM_004048	85	57°C