Evaluation of the Brain 5-HT2A Receptor Binding Index in Dogs with Anxiety Disorders, Measured with ^{123}I-5I-R91150 and SPECT

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The serotonergic system has been implicated in emotional and cognitive functions since early work. In particular, an important role has been attributed to the 5-HT2A receptor in schizophrenia, depression, eating disorders, and anxiety. The aim of the study was to evaluate the involvement of the brain 5-HT2A receptor in dogs with severe anxiety disorder, using ^{123}I-5I-R91150 and SPECT. **Methods:** SPECT was performed with the 5-HT2A receptor-specific radioligand ^{123}I-5I-R91150 to determine the 5-HT2A receptor binding index (BI) in the brains of dogs. Sixteen dogs with pathologic anxiety problems were compared with 22 normal-behaving reference dogs. **Results:** Lower 5-HT2A receptor BI was found in the left ($P = 0.001$) and right ($P = 0.002$) frontal cortices in the group of dogs with anxiety disorders than in the reference group. Right ($P = 0.022$) and left ($P = 0.048$) temporocortical BIs were also significantly lower in the dogs with anxiety disorders. Finally, the BI was significantly lower in the right occipital cortex ($P = 0.038$) of dogs with anxiety disorders than in the reference dogs. After correction for multiple comparisons ($P < 0.0056$), only the bilateral frontocortical lower BI remained significant. **Conclusion:** The findings in this study indicate that the 5-HT2A receptor is involved in the pathophysiology of anxiety disorders in dogs. The affected brain regions are in concordance with the brain regions involved in human anxiety disorders. The acquired data confirm the potential of using the dog as a natural model for investigation of the different mechanisms of anxiety disorders. In this regard, the use of dogs may contribute to the development of novel treatment approaches and new drugs for veterinary and human use.

Key Words: anxiety disorders; SPECT; serotonin (5-HT); 5-HT2A receptor; dog

J Nucl Med 2009; 50:284–289
DOI: 10.2967/jnumed.108.055731

Received Jul. 7, 2008; revision accepted Nov. 14, 2008.
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The involvement of the serotonergic system in emotional and cognitive functions has been repeatedly demonstrated in the literature. This neurotransmitter system is involved in human disorders such as schizophrenia, major depressive disorder, and disorders of impulsivity (*1,2*). Also, anxiety disorders such as posttraumatic stress disorder, panic disorder, and obsessive-compulsive disorder have been linked with dysregulation of the serotonergic system. The most robust evidence for the involvement of the serotonergic system in anxiety disorders derives from the specific efficacy of serotonergic drugs in the treatment of these disorders (*3–5*). Selective serotonin (5-HT) reuptake inhibitors (SSRIs) enhance the serotonergic transmission by inhibiting serotonin reuptake into the presynaptic neuron, causing an increased amount of serotonin to be available to stimulate pre- and postsynaptic receptors (*6*). The 5-HT1A and 5-HT2A receptors, especially, have been implicated in the antianxiety effects of long-term SSRI therapy (*7,8*). Additionally, several genetic studies revealed polymorphisms of the 5-HT2A receptor gene in patients with panic disorder (*9,10*).

Functional imaging modalities have improved the understanding of the pathophysiology of human psychiatric diseases, including one of the most prevalent mental disorders, anxiety (*11*). The highly selective 5-HT2A receptor radioligand ^{123}I-5I-R91150 used in this study has demonstrated alterations in 5-HT2A receptor binding in eating disorders and suicidal behavior (*12,13*). In addition, this tracer has been used in both healthy dogs and impulsive, aggressive dogs for the assessment of cerebral 5-HT2A receptors (*14,15*). A significantly higher 5-HT2A receptor binding index (BI) was found in the impulsive, aggressive dogs than in the reference group. Normalization of the disturbed BI and behavioral improvement was demonstrated after SSRI treatment (*16*).

The use of the dog as a model for human disease has been emphasized and strengthened by the finding that more than 400 hereditary canine diseases are known to have equivalent human diseases, including neurologic diseases. Add properties such as biologic, histologic, physiologic, and clinical similarities between human and canine diseases (*17*), and a suitable human model is at hand.

The aim of the study was to evaluate with ^{123}I-5I-R91150 and SPECT the involvement of the brain serotonin 2A receptor in dogs with severe anxiety disorders.

MATERIALS AND METHODS

Subjects

Sixteen drug-free dogs (14 male, 2 female; mean age, 37.19 ± 23.40 mo) showing anxiety behavior (freezing, inappropriate urinating, escaping, hiding, trembling, excessive salivating, scratching, etc.) and diagnosed by experienced veterinarians as having an anxiety disorder were used. These dogs were compared with a reference group of normal-behaving dogs (14 male, 8 female; mean age, 49.50 ± 21.51 mo). None of the dogs in either group had a history of neurologic disorders. Informed consent was obtained from all the dogs' owners, and all owners filled in a validated questionnaire on dog behavior (Canine Behavioural Assessment and Research Questionnaire [CBARQ]) (*18*).

All anxious dogs were selected and referred for anxiety problems by board-certified (European College of Veterinary Behavioural Medicine Diplomates) veterinarians specializing in behavioral problems (Table 1). Inclusion criteria were severe and pathologic anxiety, defined as fearful behaviors triggered by harmless stimuli or fearful behaviors at an intensity or frequency that affects the dog's safety, quality of life, or relationship with guardians (*19*).

All dogs had to be off psychopharmacologic therapy for at least 8 wk. Dogs in the reference group were included when the CBARQ scores were within the normal behavior range (*18*). Dogs were excluded if they were younger than 1 y or older than 8 y.

This study was approved by the local Ethical Committee of the Faculty of Veterinary Medicine, Ghent University.

Anesthetic Protocol

All dogs were anesthetized using a previously described protocol (*20*). In brief, sedation was obtained with a 0.1-mL intravenous injection of medetomidine hydrochloride per kilogram (Domitor; Pfizer) 30 min before the scan. General anesthesia was induced intravenously with propofol (Propovet; Abbott) and maintained with isoflurane (Isoba; Schering-Plough).

Tracers

The receptor-binding studies were performed with ^{123}I-5I-R91150. This tracer was synthesized by electrophilic substitution on the 5-position of the methoxybenzamide group of R91150, followed by purification with high-performance liquid chromatography. The ^{123}I-5I-R91150 had a radiochemical purity of more than 99% and was sterile and pyrogen-free. A specific activity of 370 GBq/μmol was obtained.

The radioligand is a 5-HT2A antagonist with high affinity (K_d = 0.11 nM) and selectivity for 5-HT2A receptors. The ligand is more selective by at least a factor of 50 for the 5-HT2A receptors than for other neurotransmitter receptors, such as the 5-HT receptors 5-HT2C and 5-HT1A, the dopamine receptors D_1 and D_2, the adrenergic receptors α_1 and α_2, and histamine receptors (*21*).

The intravenously injected activity ranged from a mean of 9.94 ± 7.36 MBq/kg of body weight in the group of dogs with anxiety disorders to a mean of 8.14 ± 3.59 MBq/kg of body weight in the reference group. Care was taken that all proceedings provoked

TABLE 1. Demographic Data and Fear Symptoms in Specific Situations of 16 Dogs with Anxiety Disorders

Dog no.	Breed	Age (mo)	Sex	Fear symptoms	Situations creating extreme fear (CBARQ)
1	LR	24	M (I)	Panic attacks, freezing, hiding	1–7
2	BG	48	M (N)	Freezing, inappropriate urinating	1–8
3	M	96	F (I)	Excessive salivating, barking, chewing	1, 9
4	BS	60	M (N)	Panic attacks, escaping, hiding, chewing	1, 5, 7
5	BuT	12	M (N)	Chewing/scratching, excessive salivating	6, 9
6	BoT	36	M (N)	Panic attacks, escaping	6, 10
7	GR	12	M (N)	Hiding, escaping	10
8	M	12	M (N)	Shivering, trembling, inappropriate urinating	2, 7, 10, 11
9	M	48	M (N)	Escaping, pulling extremely hard on leash, freezing	2, 4, 5, 7
10	MP	30	F (I)	Freezing	1, 3, 4, 11
11	B	34	M (N)	Trembling, biting	2, 9, 11
12	EB	53	M (N)	Panting, escaping, biting	2, 6, 10
13	BD	18	M (I)	Panic attacks, escaping, biting, pulling extremely hard on leash	1, 2, 5–7, 10
14	B	24	M (N)	Freezing, escaping, hiding, excessive salivating, panting	1, 2, 5–7
15	GR	66	M (I)	Escaping, hiding	1, 10
16	MS	22	M (I)	Freezing	6

LR = labrador retriever; BG = bleu de gascogne; M = mongrel; BS = berner sennen; BuT = bull terrier; BoT = border terrier; GR = golden retriever; MP = miniature pinscher; B = boxer; EB = English bulldog; BD = bordeaux dog; B = Beauceron; MS = maremma sheepdog; I = intact; N = neutered; CBARQ = 1, sudden and loud noise; 2, approached/touched by stranger; 3, heavy traffic; 4, unfamiliar object; 5, firework/thunderstorm; 6, new and unfamiliar situations; 7, wind and wind-blown objects; 8, stepped over; 9, left alone; 10, unfamiliar dog; 11, examined/treated by veterinarian.

minimal excitement of and stress to the animal. The catheter was placed intravenously 10 min before injection of the tracer to avoid tension resulting from this procedure. The radiopharmaceutical was injected 90–100 min before image acquisition. The optimal scanning time—the time when pseudo-equilibrium conditions are reached between free and bound radiotracer, necessary for semiquantification of the regional BI—was determined from 90 min onward in a previous study (*14*).

Anatomic mapping was achieved by a second SPECT acquisition reflecting the brain perfusion. These perfusion studies were performed with the ^{99m}Tc-labeled tracer *N*,*N*″-1,2-ethylene-diylbis-L-cysteine diethyl ester dihydrochloride (ECD) (Neurolite; Bristol-Myers Squibb). The intravenously injected activity ranged from a mean of 42.91 ± 28.61 MBq/kg of body weight in the group of dogs with anxiety disorders to a mean of 44.32 ± 17.02 MBq/kg of body weight in the reference group. Again, care was taken to provoke minimal excitement of and stress to the animal. The catheter was placed intravenously 10 min before injection of the tracer to avoid tension resulting from this procedure, and the radiopharmaceutical was injected 20–25 min before sedation and induction of the general anesthesia.

Scanning Procedure

The 2 functional imaging examinations, brain perfusion SPECT and brain 5-HT2A receptor–binding SPECT, were performed with an interval of at least 1 wk but no longer than 3 wk. All dogs were positioned prone. The detectors of the triple-head γ-camera (Triad; Trionix) were positioned as close as possible to the dog's head. A preformed head-resting cushion was used to minimize intraindividual positioning variability. In addition, the height of the imaging table, the radius of rotation, and the depth position of the dog's head in relation to the camera were noted for each subject, and the same measurements were used for the second SPECT scan.

The triple-head γ-camera was equipped with low-energy ultra-high-resolution parallel-hole collimators (tomographic resolution, 9-mm full width at half maximum). Data for the 5-HT2A receptor scan were acquired for 30 min in step-and-shoot mode (90 steps, 20 s/step, 4° steps) on a 128 × 128 matrix. Data for perfusion imaging were acquired for 20 min in step-and-shoot mode (120 steps, 10 s/step, 3° steps) on a 128 × 128 matrix. Images were reconstructed with filtered backprojection and application of a Butterworth filter (cutoff, 1.6 cycles/cm; order, 10). Pixel size was 1.72 mm.

Image Analysis

The emission ^{123}I-5I-R91150 ligand data were matched with the emission ^{99m}Tc-ECD perfusion data. This data fitting was performed with multimodality software (version 5.0; Nuclear Diagnostic AB). The software, displaying images in a dual-window setting, allows for manual coregistration by providing tools for scaling, rotating, and translating images in all 3 dimensions. Images were analyzed using region-of-interest (ROI) analysis. ROIs were drawn over the frontal, temporal, parietal, and occipital cortices (left and right). The cerebellum and the subcortical region were also included. For all areas, ROIs were first drawn and oriented on the perfusion images and further manually positioned on the 5-HT2A receptor ligand images. This latter readjustment, consisting only of minor changes of position (and not of shape or size) of the ROIs, was based on the visual examination of our perfusion-based position of the ROIs on the 5-HT2A receptor–binding images. In the cases readjustment was necessary, changes in position never required more than 2 pixels (maximum, 3.4 mm) on the *x*-, *y*-, or *z*-axes. The uptake in the global cerebellum (a 5-HT2A receptor–poor region) (*22*) was used as a reference for nonspecific binding in addition to free ligand. Radioactivity measured in the cortical areas was assumed to represent the total activity (i.e., specific + nonspecific activity + free ligand). The BI was operationally estimated as ([counts per pixel in regional cortex] − [counts per pixel in cerebellum])/[counts per pixel in cerebellum]). This BI is proportional to the in vivo density of available receptors under pseudo-equilibrium conditions (*22*).

Statistical Methods

An independent Student *t* test was applied to evaluate the equality of age, weight, and injected dose per kilogram of body weight of ^{123}I-5I-R91150 between the 2 groups. Sex difference and gonadal status were analyzed using the Fisher exact test. Radioligand data were analyzed using the nonparametric Mann–Whitney *U* test. Level of significance was set at a *P* value of less than 0.05 for all analyses. Another *P* value, corrected for multiple comparisons using the Holm's sequentially rejective Bonferroni correction, was also proposed for the radioligand data ($\alpha' = 1 - [1 - \alpha]^{(1/N)} = 0.0056$). SPSS software (version 15; SPSS Inc.) was used to perform the analyses.

RESULTS

Data are mean ± SD.

Subject Population

Only 1 of the 16 dogs had a history of treatment with psychotropics, but not in the 8 wk preceding the study. There was no statistical age difference between the dogs with anxiety disorder (age, 37.19 ± 23.40 mo) and the normal-behaving reference dogs (age, 49.50 ± 21.51 mo) (Table 2). For that reason, no age correction was applied. Neither sex nor gonadal status was significantly different between the 2 groups.

Serotonin-2A Receptor BI

The injected dose of the radiopharmaceutical ^{123}I-5I-R91150 per kilogram of body weight was not significantly different between the 2 groups (Table 2). The injected dose of the radiopharmaceutical ^{99m}Tc-ECD per kilogram of body weight was also not significantly different between the 2 groups ($P = 0.17$).

Using the conventional *P* value of less than 0.05, we found a significantly lower BI in the left ($P = 0.001$) and right ($P = 0.002$) frontal cortices in the dogs with anxiety disorders than in the reference dogs (Table 3). Left ($P = 0.048$) and right ($P = 0.022$) temporocortical BIs were also significantly lower in dogs with anxiety-related problems. Finally, the dogs with anxiety disorders had significantly lower 5-HT2A receptor BI in the right occipital cortex ($P = 0.038$) than did the reference dogs.

Only the decreased 5-HT2A BI in the left and right frontocortical areas–remained significant using a *P* value of less than 0.0056, corrected for multiple comparisons.

TABLE 2. Demographic and Experimental Data of Anxious and Reference Dogs

Data	Anxious dogs (n = 16)	Reference dogs (n = 22)	Statistics	
			$t/\chi 2$	P
Mean age ± SD (mo)	37.19 ± 23.40	49.50 ± 21.51	t = 1.68	0.07
Mean weight ± SD (kg)	28.53 ± 14.12	25.84 ± 8.82	t = −0.74	0.50
Sex (M/F)	14/2	14/8	$\chi 2$ = 2.72	0.14 (FE)
Gonad status (I/N)	6/10	9/13	$\chi 2$ = 0.05	0.99 (FE)
Mean injected dose (± SD) of ^{123}I-R-91150 per kilogram	9.94 ± 7.36	8.14 ± 3.59	t = 0.86	0.40

I = intact; N = neutered; FE = Fisher exact test.

The mean age of the reference dogs was higher than that of the dogs with anxiety disorders, albeit statistically not significant. A former study demonstrated that older age is associated with a decrease in 5-HT2A BI (*23*). Therefore, an underestimation of the lower BI in the group with anxiety disorder cannot be completely excluded.

Brain perfusion changes were excluded from being the origin for the registered altered 5-HT2A receptor BIs because no overlap was noticed between the 2 datasets. The only common decrease in perfusion and 5-HT2A receptor BI was noticed in the left frontal cortex, and this decrease disappeared when we applied the correction for multiple comparisons (Table 4).

DISCUSSION

The major findings of this study are a lower mean serotonin-2A BI in the frontal and temporocortical regions (both left and right) and right occipital cortex in the dogs with anxiety disorders than in the reference dogs.

Basic evidence for the involvement of the 5-HT2A receptor, in particular in anxiety disorders, can be found in the anxiolytic effect of 5-HT2A receptor agonists (such as [(±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane]) and the anxiogenic effect of 5-HT2A receptor antagonists in rodents (*24*). PET reduced binding of ^{18}F-altanserin, and ^{18}F-setoperone was found in several cortical regions in patients who were depressed, indicating a reduced 5-HT2A receptor-binding potential (*25,26*). After successful treatment with SSRIs, an increased 5-HT2A receptor binding was found on PET (*27*). A reduced 5-HT2A BI was also demonstrated using SPECT in patients with anxiety and mood disorders (*28*). Furthermore, the recently described decreased inhibition in anxiety-related behaviors in 5-HT2A receptor knockout mice (*29*) is also in concordance with our results.

The reason for the lower BI index may be that the receptor is downregulated because of serotonergic activation in anxiety disorders in dogs. Evidence of an increased serotonin turnover in patients with depression and panic disorders was provided in a study that reported an increased 5-hydroxyindoleacetic acid level in the internal jugular venous plasma (*30*). Using in vivo microdialysis, we demonstrated in rats that conditioned fear stress resulted in increased extracellular serotonin in the medial frontal cortex. This increased release was followed by a resolution of freezing behavior (*31*). These findings suggest that facilitation of serotonergic transmission is a natural defense mechanism against anxiety and could also explain the efficacy of SSRIs in anxiety disorders. However, because we did not measure 5-hydroxyindoleacetic acid in this study and because microdialysis was beyond the scope of this study, this explanation for the decreased binding is only speculative.

Peremans et al. investigated the 5-HT2A receptor in impulsive aggressive dogs. They found a higher 5-HT2A BI in all cortical regions in the impulsive, aggressive dogs than in the normal-behaving dogs (*15*). The highest difference was found in the frontal cortex ($P < 0.001$).

TABLE 3. BI in Anxious and Reference Dogs

ROI	Anxious dogs (mean ± SEM)	Reference dogs (mean ± SEM)	Statistics z (Mann–Whitney U test)
Left frontal cortex	1.52 ± 0.05	1.78 ± 0.05	3.01*
Right frontal cortex	1.54 ± 0.05	1.78 ± 0.04	3.28*
Left temporal cortex	1.36 ± 0.06	1.48 ± 0.05	1.98†
Right temporal cortex	1.39 ± 0.06	1.53 ± 0.04	2.29†
Left parietal cortex	1.39 ± 0.06	1.34 ± 0.08	0.49
Right parietal cortex	1.38 ± 0.06	1.41 ± 0.08	0.49
Left occipital cortex	1.34 ± 0.05	1.45 ± 0.06	1.32
Right occipital cortex	1.30 ± 0.05	1.48 ± 0.06	2.08†
Subcortical region	1.19 ± 0.05	1.20 ± 0.08	0.20

*$P < 0.0056$ (Holm's Bonferroni correction).
†$P < 0.05$.

TABLE 4. Perfusion and 5-HT2A BI at $P < 0.05$ and $P < 0.0056$

	$P < 0.05$		$P < 0.0056$	
ROI	Perfusion	5-HT2A BI	Perfusion	5-HT2A BI
Left frontal cortex	↓	↓	–	↓
Right frontal cortex	–	↓	–	↓
Left temporal cortex	↑	↓	–	–
Right temporal cortex	↑	↓	↑	–
Left parietal cortex	–	–	–	–
Right parietal cortex	–	–	–	–
Left occipital cortex	–	–	–	–
Right occipital cortex	–	↓	–	–
Subcortical region	↓	–	↓	–

↓ = significant decrease; ↑ = significant increase; – = no significant change between both anxious and reference groups.

Also, SSRIs are effective antidepressive drugs only after a certain delay. That is thought to be due to the time needed for 5-HT1A autoreceptors to desensitize. Administration of a drug combining a serotonin reuptake inhibitor and a 5-HT1A autoreceptor antagonist is proven to act more quickly, without the long delay to therapeutic onset (*32*). By combining these findings, one can hypothesize that increased serotonin due to 5-HT1A autoreceptor deactivation may lead to a downregulation of the postsynaptic 5-HT2A receptor.

Another possible hypothesis is that this lower BI is trait-related and that low 5-HT2A receptor signaling may be an indicator for vulnerability to anxiety disorders. Several recent genetic studies demonstrated an association between different single nucleotide polymorphisms of the gene coding for the 5-HT2A receptor (HTR2A) and panic disorder, suggesting an important role for the HTR2A in the development of panic disorder (*9,10*). Another study suggests a similar association concerning the development of social anxiety disorder (*33*). However, to our knowledge this has not yet been investigated in dogs with anxiety disorders.

In addition to a serotonergic imbalance, the involvement of the noradrenergic system in depression and anxiety disorders is also an essential factor, and interplay between the 2 neurotransmitter systems is well recognized (*7,34*). The highest concentration of norepinephrine is found in the locus coeruleus and acts mostly inhibitory on the raphe nuclei, the region with the majority of serotonergic neurons. Via the 5-HT receptors, the raphe nuclei also has inhibitory action on the locus coeruleus. In general, damage to the 5-HT neurons has been associated with increased firing of noradrenergic neurons, and long-term treatment with SSRIs provoked a progressive decrease in the firing activity of noradrenergic neurons (*7*). In particular, activation of the 5-HT2A receptor suppressed the firing rate of noradrenergic neurons (*7,8*), and it can be hypothesized that low signaling of the 5-HT2A receptor may result in increased firing of noradrenergic neurons.

The robust finding that the 5-HT2A receptor BI in the frontal cortex is deficient in anxious dogs is consistent with the major involvement of the 5-HT2A receptor BI in fear and anxiety. Lack of control of the ventral medial prefrontal cortex over a hyperactive amygdala (*35*) has been suggested in anxiety disorders such as phobias (*36*). In rats, lesions of the dorsal medial prefrontal cortex showed an exaggerated freezing response to a conditioned stimulus (*37*), and microdialysis demonstrated increased serotonin levels in the conditioned-fear stress paradigm in the medial frontal cortex (*31*). In this study, because of resolution limits, identification of frontal cortex subdivisions was not attempted, and global frontocortical alterations in radioligand BI were recorded.

The decreased 5-HT2A receptor BI registered in the temporal area could be explained by the involvement of the hippocampus, a brain area that can be found near the temporal area (*38*). The hippocampus, centerpiece of the limbic system, is known to play a critical role in learning and memory but is also involved in the fear network of the brain (*6,11*).

Finally, on the basis of the results of this study, it is not possible to conclude whether the lower density of 5-HT2A receptors is causal to, or a consequence of, anxiety disorders in dogs.

Methodologic Considerations

General anesthesia was required to perform these examinations. The use of anesthesia can alter global cerebral blood flow and possibly influence the pseudo-equilibrium state of the radioligand. Indirect effects by medetomidine (an α_2 agonist) can be expected through interaction of the α_2 autoreceptor on serotonergic terminals. However, the interval between sedation and scanning was less than 10 min, a time during which occurrence of these effects may be considered negligible, albeit not impossible. Different studies have also pointed out an effect of volatile anesthetics (e.g., isoflurane) on the serotonergic system (*39,40*). Mukaida et al. described a decreased frontocortical release of the neurotransmitter serotonin in rats during isoflurane administration (*39*). However, we removed these interactions between anesthetics and radioligand binding by adhering strictly to the same anesthetic protocol in both groups.

CONCLUSION

A significantly lower 5-HT2A receptor radioligand binding was found in regions assumed to play an important role in the pathophysiology of anxiety disorders. The acquired data confirm that the dog's brain has value as a model for investigating the different mechanisms of anxiety disorders in humans and may contribute to the development of novel treatment approaches and new drugs for veterinary and human use.

ACKNOWLEDGMENTS

We thank Tiny De Keuster, Diplomate ECVBM, for referral of cases. This study was supported by a scientific research grant from the Ghent University Special Research Fund.

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