

GENERATION AND CHARACTERIZATION OF A HUMAN BRADYKININ
RECEPTOR B1 TRANSGENIC RAT AS A PHARMACODYNAMIC MODEL

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Running Title: Human Bradykinin Receptor B1 Transgenic Rat

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Text Pages: 37
Number of Tables: 2
Number of Figures: 6
References: 37
Number of Words in Abstract: 247
Number of Words in Introduction: 605
Number of Words in Discussion: 1468

Abbreviations: B1R, bradykinin receptor B1; B2R, bradykinin receptor B2; CHO, Chinese hamster ovary; Compound A, *N*-{2-[4-(4,5-dihydro-1*H*-imidazol-2-yl)phenyl]ethyl}-2-[(2*R*)-1-(2-naphthylsulfonyl)-3-oxo-1,2,3,4-tetrahydroquinoxalin-2-yl]acetamide; Compound B, (3*R*)-3-(3,4-dichlorophenyl)-*N*-{(1*R*)-1-[4-(4,5-dihydro-1*H*-imidazol-2-yl)benzyl]-2-oxo-2-pyrrolidin-1-ylethyl}-3-[(2-naphthylsulfonyl)amino]propanamide; Compound C, 2-[(2*R*)-1-(3,4-dichlorophenyl)sulfonyl]-3-oxo-1,2,3,4-tetrahydroquinoxalin-2-yl]-*N*-{2-[4-(4,5-dihydro-1*H*-imidazol-2-yl)phenyl]ethyl}acetamide; Compound D, 3-[(2,2-difluorocyclopropyl)carbonyl]amino)-*N*-{(1*R*)-1-[2'-(methoxycarbonyl)-5'-methylbiphenyl-4-yl]ethyl}-4-methylpyridin-2-aminium chloride; Compound E, *N*-[4-chloro-2-[(1*R*)-1-[3'-fluoro-2'-(3-methyl-1,2,4-oxadiazol-5-yl)biphenyl-4-yl]ethyl]amino)pyridin-3-yl]-3,3,3-trifluoropropanamide; LPS, lipopolysaccharide; NSE, Neuron-specific enolase

Abstract:

Antagonists of the B1 bradykinin receptor (B1R) offer the promise of novel therapeutic agents for the treatment of inflammatory and neuropathic pain. However, the *in vivo* characterization of the pharmacodynamics of B1R antagonists is hindered by the low level of B1R expression in healthy tissue and the profound species selectivity exhibited by many compounds for the human B1R. To circumvent these issues we generated a transgenic rat expressing the human B1R under the control of the neuron-specific enolase (NSE) promoter. Membranes prepared from whole brain homogenates of heterozygous transgenic rats indicate a B1R expression level of 30-40 fmol/mg; there is no detectable B1R expression in control non-transgenic rats. The pharmacological profile of the B1R expressed in the transgenic rat matches that expected of the human, but not the rat receptor. The mapping of the transgene insertion site to rat chromosome 1 permitted the development of a reliable assay for the identification of homozygous transgenic rats. Significantly, homozygous transgenic rats express two-fold more B1R than heterozygous animals. Autoradiographic analyses of tissue sections from transgenic rats reveal that the B1R is broadly expressed in both the brain and spinal cord. The human B1R expressed in the transgenic rat functions in an *in vitro* contractile assay and thus has the potential to elicit a functional response *in vivo*. Using the humanized B1R transgenic rat, an assay was developed that is suitable for the routine evaluation of a test compound's ability to occupy the human B1R in the central nervous system.

The bradykinin B1 (B1R) and B2 (B2R) receptor subtypes mediate the action of the kinin peptides which are liberated from the large protein precursor kininogen in response to environmental stimuli (reviewed by (Bock et al., 2003; Couture et al., 2001; Marceau et al., 1998)). The B2 receptor is constitutively expressed in a number of tissues and mediates many of the acute actions of kinins. In contrast, the B1R is induced upon tissue injury and is expressed at very low levels in healthy tissue. This is consistent with the initial discovery of the B1R as mediating a de novo pharmacological response that appeared in vascular tissue preparations following an in vitro incubation (Regoli et al., 1978). Subsequent work defined a cytokine network that is involved in mediating induction of the B1R in response to tissue damage. However, recent work has discovered exceptions to the general notion that the B1R is not present in normal tissue; in particular B1R appears to be expressed at low levels in healthy central nervous system (CNS) tissue of rodents and primates (Ma and Heavens, 2001; Ma, 2001; Shughrue et al., 2003; Wotherspoon and Winter, 2000). The low level B1R expression in the CNS is increased in response to tissue injury (Fox et al., 2003; Mason et al., 2002). The characterization of the B1R knockout mouse provided compelling evidence for the B1R expressed in the CNS playing a significant role in mediating nociception (Pesquero et al., 2000). Additional studies support these initial observations (Ferreira et al., 2002; Fox et al., 2003); therefore it is desirable to identify B1R antagonists with both central and peripheral action as potential analgesic agents.

Molecular cloning of the B1R and B2R subtypes revealed that they are G-protein coupled receptors that are relatively distinct, only 38% identical at the amino acid level (McEachern et al., 1991; Menke et al., 1994; Hess et al., 1992). The cloning of the B1R

from a number of species demonstrated that this receptor is not highly conserved across species, the conservation from rodent to man being only 73% amino acid identity (Jones et al., 1999; Hess et al., 1996; MacNeil et al., 1995; Hess et al., 2001; Ni et al., 1998), by comparison the angiotensin II type I receptor is 94% identical between rodent and man. The species divergence observed for B1R at the amino acid level results in pharmacological differences, as indicated in several studies examining the affinity of the B1R from different species for kinin peptides (Hess et al., 1996; MacNeil et al., 1995; Jones et al., 1999; Regoli et al., 1997). Since B1R species differences are observed for the natural ligands, it is expected that species differences will be evident for synthetic compounds. Indeed, species differences are observed in the binding affinity of B1R for both synthetic peptide and nonpeptide compounds (Su et al., 2003; MacNeil et al., 1997).

Efficient development of therapeutic compounds is dependent upon the ability to readily evaluate the pharmacodynamic properties of synthetic compounds in preclinical models. However, the determination of the pharmacodynamic properties of human B1R selective compounds is encumbered by two issues. The first being the very low levels of B1R expression in healthy tissue. Though it may be possible to elicit tissue damage and induce the B1R to levels sufficient for pharmacodynamic studies, this introduces an additional level of complexity to the experiment. Even if successful, induction of the endogenous B1R would not address the species selectivity of synthetic compounds. To address these issues, we report the generation and characterization of a transgenic rat in which the human B1R is constitutively over-expressed under the control of the rat neuron specific enolase (NSE) promoter.

Materials and Methods:

Generation of transgenic rats

Rat genomic DNA (100 ng/50 ul reaction) was used as a template to generate a PCR fragment containing the rat NSE promoter. The portion of the promoter used begins with the sequence 5'-TGAGCTCCTCCTCTGCT-3' and ends with the sequence 5'-GTCTGCAGTCCTCGAG-3'. This promoter was fused to CMV intronA by overlap extension PCR (Horton et al., 1990). A PCR product containing the human B1R coding sequence and the bovine growth hormone polyadenylation signal was obtained from a vector, pcDNA3, containing the human B1 receptor coding sequence. This amplified product was connected to the NSE/CMV intron A product by overlap extension PCR. The resulting 4.1 kb product was subcloned into pBlueScript. To provide DNA for micro-injection the 4.1 kb transgenic construct was excised from the vector backbone, gel purified, and resuspended in 10 mM Tris pH 7.4, 0.1 mM EDTA at a 50 ng/ul concentration. The purified NSE promoter/CMV intronA/human B1/BGH polyA fragment was provided to DNX Transgenic Sciences (currently Xenogen Corporation) in Princeton, NJ under contract for the generation of transgenic rats. Standard methodology was utilized to inject the construct into Sprague-Dawley rat eggs to create transgenic rat lines (Wagner et al., 1981).

Genotyping of transgenic rats

A PCR genotype assay to identify transgenic rats was developed using the forward primer 5' CAGAAGAAGATGCAGGCAGC-3' and the reverse primer 5'-GAGATGATAAATGTCGGCAGC-3' to produce a diagnostic fragment of 342 nucleotides. A Southern blot assay was established using a 701 nucleotide product that

was isolated using the forward primer 5'-AATCTCGGGTACGTGTTCCG and reverse primer 5'-TTGGCCAGGTAGATTTCTGC-3'. The product was radiolabeled with [α -³²P]dCTP by random prime labeling (Roche Applied Science, Indianapolis IN) and used to probe genomic DNA digested with *Eco* RI. Hybridization was performed in 0.25 M Na₂HPO₄, 6.5% SDS, and 10% dextran sulfate at 65°C overnight. The blot was washed with a final wash of 0.1X SSC 0.1% SDS for 30 minutes at 60°C and exposed to film.

Fine mapping of line 4 transgene integration site

Genomic DNA was prepared from tissue of a heterozygous transgenic rat from line 4. The genomic DNA was partially digested with restriction endonuclease *Sau* 3A1 and cloned into the superCOS I vector according to the manufacturer's instructions (Stratagene, La Jolla CA). Cosmid clones were screened by standard in situ hybridization of bacterial colonies using the radiolabeled probe consisting of 701 nucleotides described above.

The genomic DNA sequence upstream of the transgene insertion site was utilized to design forward, 5'-GAGGTGAAGGCCACATTTTCTAGC, and reverse 5'-ATGGGGAAGGAGTTGATGAAAGGTAGCC, PCR primers. Using the cosmid DNA template and standard PCR procedures these primers generate a product of 922 nucleotides. This fragment of 922 nucleotides serves an external probe that was radiolabeled and used in Southern blot analysis to discern wild type from transgenic chromosomes.

Recombinant cell lines

The human bradykinin B1 receptor coding sequence subcloned into a mammalian expression vector were introduced into Chinese Hamster Ovary (CHO) cells as

previously described (Kunapuli et al., 2003). Clonal cell lines were obtained and propagated by standard procedures.

Autoradiographic analysis

For autoradiographic analysis of human B1 receptor expression, tissues were removed from transgenic rats, frozen on dry ice powder, and stored at -70°C. Coronal sections of the brain and the transverse sections of the spinal cord were prepared with cryostat (Leica, CM3050) set at 20 micron. The frozen sections were stored at -70°C. For analysis, frozen sections were warmed at room temperature (RT) for 15 minutes; then followed by 15 minutes preincubation in the buffer without radioligand at RT. After preincubation, the sections were transferred to the incubation buffer, and incubated for 90 minutes at RT. Total binding, both non-specific and specific, was determined by incubating in buffer containing 10 pM [³⁵S]compound A (*N*-{2-[4-(4,5-dihydro-1*H*-imidazol-2-yl)phenyl]ethyl}-2-[(2*R*)-1-(2-naphthylsulfonyl)-3-oxo-1,2,3,4-tetrahydroquinoxalin-2-yl]acetamide). An adjacent section was utilized to determine non-specific binding, which was incubated in buffer containing 10 pM [³⁵S]compound A and 200 nM of a non-peptide receptor antagonist, compound B (3*R*)-3-(3,4-dichlorophenyl)-*N*-{(1*R*)-1-[4-(4,5-dihydro-1*H*-imidazol-2-yl)benzyl]-2-oxo-2-pyrrolidin-1-ylethyl}-3-[(2-naphthylsulfonyl)amino]propanamide) that exhibits high affinity for the human B1 bradykinin receptor. Following the 90 minute incubation, the sections were washed three times, 3 minutes each, in buffer, rinsed in de-ionized H₂O for 30 seconds at 4°C, and then dried by air blower at RT. The sections were placed against Fuji imaging plates, and exposed for a week at RT. The plates were scanned with Fuji

PhosphorImager BAS 5000, and the images were analyzed with MCID M5 software (Imaging Research, St. Catharines, Ontario Canada).

Functional assay in isolated rat ileum

Isolated rat ileum longitudinal muscle strips were mounted on tissue holders and placed in 5 ml tissue baths containing Krebs solution consisting of 118 mM NaCl; 4.7 mM KCl; 2.5 mM CaCl₂; 1.2 mM KH₂PO₄; 1.2 mM MgSO₄; 25 mM NaHCO₃; and 11 mM dextrose. The tissue baths were kept at 37°C and aerated with 95% oxygen and 5% carbon dioxide. One gram of tension was applied to the tissues. The tissues were allowed to equilibrate for 3 hr and were washed every 20 min. The expression of the endogenous rat B1R was blocked by the addition of 70 uM cycloheximide during the 3 hr equilibration period. Contractile tension was recorded using Statham force transducers and 7700 series Hewlett Packard recorders.

Test compound administration

Transgenic rats of either sex were placed in an induction chamber and anesthetized with isoflurane under a Flow Sciences hood. Once anesthetized, the rat was placed on a circulating water warming blanket (Gaymar T-pump) and anesthesia was maintained using 2% isoflurane by means of a nose cone. The tail vein was cannulated with a 25G winged infusion set-up connected to a syringe containing either test compound or vehicle. The desired dose of test compound was administered. At the experimental end-point, a blood sample was taken, the rat was euthanized, and tissues were removed for subsequent assays.

Ex vivo homogenate binding assay

For homogenate binding assays, thirty-five milligrams of frozen brain (cerebral cortex or cerebellum) or spinal cord was homogenized with a Polytron, in a large volume of ice-cold assay buffer (20 mM HEPES, 120 mM NaCl, 5 mM KCl, pH 7.4) and transferred to two chilled centrifuge tubes. To pellet membranes, the tubes were centrifuged for 10 minutes at 75,000xg in a rotor pre-cooled to 4°C. The supernatant was discarded, each tube was rinsed with 20 ml ice-cold buffer, and the pellets were homogenized in ice-cold assay buffer. The homogenate was pooled and added to a tube containing the radiotracer, 20 pM of [³⁵S]compound A, in each tube containing 0.5 ml room temperature assay buffer. Nonspecific binding was determined by adding homogenate to tubes containing the radiotracer and 100 nM of the unlabeled compound A. At set time points (1, 2, 4, 6, 8, 10 minutes) the contents of three tubes are filtered over individual 25 mm GF/B filters presoaked in 0.05% Triton X-100. The filtration step was performed by adding 4 ml ice-cold assay buffer to each of the three replicate tubes, pouring the contents over the filters, and washing each filter two times with 4 ml ice-cold buffer. A Hoeffler FH 225V filtration manifold was used for the filtration. The nonspecific binding tubes were similarly filtered after completion of the time points. Filters are transferred to 5 ml scintillation vials and counted after soaking 10 hours in 3 ml Beckman Ready Safe scintillation fluid.

Results:

Generation of human B1 transgenic rats

A DNA construct was prepared containing the rat NSE promoter, CMV intron A, the human B1R coding sequence, and the bovine growth hormone polyadenylation signal (Figure 1A). An 1806 nucleotide fragment of the rat NSE promoter, including the

primary transcriptional start site and exon 1, was obtained by PCR from rat genomic DNA and fused to cytomegalovirus (CMV) intron A. CMV intron A is reported to contain enhancer elements which may improve expression (Xu et al., 2001); in addition, the presence of an intron in the construct may enhance the stability and processing of the transcript derived from the artificial gene (Huang and Gorman, 1990). CMV intron A was connected to the 5' end of human B1R exon 3, which contains the entire coding sequence for the receptor. The transgene construct was completed by the addition of the bovine growth hormone poly adenylation signal.

Xenogen Biosciences, Inc. used standard microinjection techniques to introduce the NSE/B1R transgene construct into Sprague Dawley rat eggs. Genomic DNA was obtained from 26 progeny of the transgene injections, PCR and Southern blot analysis indicated that 5 founder rats contained at least one copy of the transgene (Figure 1B). Founder rats 4, 14, 15, and 23 contain more than 10 copies of the transgene, with the highest copy number being detected in founder 14.

Mapping of the transgene integration site in line 4

The insertion site of the human B1R transgene was determined in line 4. The primary motivation for mapping the transgene integration site was to develop a reliable assay for the detection of homozygous transgenic rats. Screening of a cosmid library prepared from line 4 genomic DNA identified cosmid clone 19. DNA sequence analysis of the ends of cosmid clone 19 DNA revealed that one end was identical to rat genomic sequence contained in clone CH230-6B11 (GenBank Accession number AC097387) and the other end was identical to the human B1R transgenic construct. Clone CH230-6B11 maps to rat chromosome 1 and contains the zona pellucidae glycoprotein 1 (ZP1) gene.

Cosmid clone 19 was digested with the restriction enzyme *Dra* I and the resulting fragments were end-sequenced. This strategy identified a 1.7 kb fragment that matched rat genomic DNA sequence found in clone CH230-6B11 at one end and the transgene construct at the other end. Complete DNA sequence analysis of the 1.7 kb *Dra* I fragment revealed the location of the transgene integrations site to be approximately 20.5 kb downstream of the 3' end of the ZP1 gene and 1.2 kb upstream of the first exon of LOC246216, a neuronal differentiation-related gene. The NSE promoter directs transcription in the opposite direction of the promoter for LOC246216 and in the same direction as the ZP1 gene. The initial construct unit, present in the transgene insertion, lacked the first 40 bases of the NSE promoter; whereas the adjacent copy, assembled in a head to tail fashion, appeared to be intact. Detailed analysis of the transgene insertion site revealed 266 nucleotides at the site of insertion that are “scrambled” as a consequence of DNA from several sources, including the linear construct, being utilized to repair the gap opened in the process transgene integration.

The fine mapping of the transgene insertion site permitted the development of a Southern blot genotype assay using a 922 nucleotide probe upstream of the transgene insertion site to readily discern wild type, heterozygous, and homozygous transgenic rats. Analysis of the DNA sequence contained for clone CH230-6B11 in GenBank Accession number AC097387 predicted that digestion with the restriction endonuclease *Dra* I would generate a fragment of 3.62 kB that contains the probe sequence. Accordingly, Southern blot analysis of *Dra* I digested genomic DNA and the 922 nucleotide probe yielded a single band of the expected size (Figure 1C; lane 1). Southern blot analysis of *Dra* I digested genomic DNA from a line 4 transgenic positive rat yielded bands of 3.6

kB and a 1.7 kB fragment, as predicted (Figure 1C; lane 2). Finally, interbreeding of heterozygous transgenic rats yielded the three expected genotypes, including homozygous transgenic rats with a single 1.7 kB fragment detected by Southern blot analysis (Figure 1C; lane 3).

B1R expression levels in different transgenic rat lines

Transgenic progeny were derived from four of the five founder rats and evaluated for expression of B1R. The expression level of the transgenic rat lines was determined by saturation binding analysis using the radioligand [³H]des-Arg¹⁰,Leu⁹kallidin (DALK) on membranes prepared from tissue homogenates. The highest level of B1R expression was found in the brain of heterozygous transgenic rats from line 4 (Table 1, Figure 2A). In contrast, B1R expression in wild type rats is below the level of detection with [³H]DALK. Relative to transgenic line 4, transgenic lines 14 and 15 exhibited an approximately 10 and 5-fold lower level of B1R expression, respectively, and were not pursued further (Table 1). A single animal from line 23 exhibited an expression level approximately one-half that of line 4, however the analysis of two additional animals yielded an expression level 10-fold lower than line 4. The lower expression level and the potential for animal-to-animal variability resulted in this line not being pursued further. A comparison of the B1R expression level in the different transgenic lines with the genomic Southern indicated a lack of correlation between B1R expression and transgene copy number.

Human B1R transgenic rat line 4 was selected for further characterization. Eighteen line 4 heterozygous transgenic rats, ranging in age from 7 weeks to 4 months were tested for the level of expression of B1R in the brain (Table 1). The expression was

consistent across age and sex (data not shown). These results indicated minimal animal-to-animal variation, enabling the development of a robust radioligand binding assay. In addition to the central nervous system tissues, whole tissue homogenates from heart and ileum of transgenic line 4 were examined. In the peripheral tissues specific binding was detected, but the expression level was greater than 10-fold lower than that observed in the brain (data not shown).

The characterization of the homozygous line 4 transgenic animals provided good evidence for a proportional gene dosage effect with approximately 2-fold higher human B1R expression levels in whole brain homogenates prepared from homozygous rats, relative to heterozygous rats (Table 1). Similar results were obtained with spinal cord tissue (data not shown). The identification of homozygous transgenic rats allowed the establishment of homozygous breeding pairs. The homozygous transgenic rats were fertile and produced normal litter sizes. There was no overt behavioral phenotype of either heterozygous or homozygous human B1R transgenic rats upon general observation (see also Discussion) nor was there any significant difference in body weight between genotypes when measured between 6-8 weeks of age.

Pharmacology of the B1R detected in transgenic rats

The pharmacological profile of the B1R was evaluated to determine whether the B1R expressed in the transgenic rat matches that of the human or rat B1R. The affinity of the human B1R for des-Arg¹⁰kallidin derived peptides is significantly higher than that found for rodent receptors (Fathy et al., 2000; Hess et al., 1996; Jones et al., 1999; Ni et al., 1998). This difference was observed in the binding affinity of DALK for cloned human and rat B1R heterologously expressed in CHO cells, with the human receptor

exhibiting 100-fold higher affinity (Table 2). The mean K_d of the B1R detected in the brain from each of the four transgenic rat lines for [^3H]DALK closely matched that of the human, but not the rat, B1R (Figure 2A, Table 1). This data provided initial evidence that the B1R detected in the transgenic rat is of human origin.

As indicated above, a number of synthetic compounds exhibit selectivity for the human B1R relative to the rat B1R (MacNeil et al., 1997; Su et al., 2003). To further evaluate the pharmacological properties of transgenic rat line 4, a set of novel human selective compounds was tested (Figure 2B). The affinity of these compounds, as determined by competition binding analysis, for the B1R expressed in the brain of transgenic rat line 4 closely matched that of the recombinant human B1R expressed in CHO cells (Table 2). In contrast, the pharmacology of the B1R in the transgenic rat did not match that of the recombinant rat B1R expressed in CHO cells (Table 2). Therefore, based on these results we concluded that the B1R detected in the transgenic rat brain is the human B1R.

Localization of CNS expression of the human B1R transgene

The location of human B1R expression in the CNS of transgenic rat lines was studied by autoradiography using either the nonpeptide radioligand [^{35}S]compound A (Figure 3) or the peptide radioligand [^3H]DALK (Figure 4). The relative expression level, as determined by specific binding of the radioligands, was similar to that observed for the membrane binding of brain homogenates, i.e. the highest level of expression is observed with line 4. There was no specific radioligand binding in wild type rats (Figure 3). The human B1R is widely expressed throughout the brain in all of the transgenic

lines examined, which is consistent with the pattern anticipated for the pan-neuronal NSE promoter.

A detailed analysis of the radioligand binding in transgenic line 4 revealed specific brain regions with relatively higher B1R expression levels; these included the cerebral cortex, hypothalamus, thalamus, cerebellum, substantial nigra, interpeduncular nucleus, nucleus of solitary tract, periaqueductal gray, and pontine nucleus (Figure 4). In addition to the brain, autoradiography was performed on transverse sections of the spinal cord, with the highest level of expression being observed in the dorsal horn (Figure 4). Currently, it is unclear why higher expression is observed in these specific regions, though chromosomal context of the transgene insertion site is a likely factor.

Functional activity of the human B1R transgene

Radioligand binding studies determined that the B1R transgenic rat expressed a receptor that has the pharmacological properties of the human B1R. To determine whether the human B1R was capable of eliciting a functional response, the ability of agonists to contract the smooth muscle of the ileum was examined. This tissue preparation was selected based upon previously reported assays for the detection of the endogenous rat B1R (Meini et al., 1996; Ueno et al., 2002). The contraction of the rat ileum in response to B1 agonists is dependent upon the de novo synthesis of B1R and thus can be blocked by inhibitors of protein synthesis such as cycloheximide (Meini et al., 1996; Ueno et al., 2002). This finding was replicated in non-transgenic rats, in which DAK and DABK elicited a contractile response with a mean EC₅₀ of 6.4 nM (95% confidence interval of 3.7 to 11 nM) and 2.6 nM (95% confidence interval of 1.7 to 3.9 nM), a rank order of potency similar to those previously reported (Meini et al., 1996).

This response to B1 agonists in the non-transgenic rat was completely blocked by 70 μ M cycloheximide (Figure 5A), thus cycloheximide was included during tissue incubation of transgenic rat ileum to block the expression of the rat B1R. Accordingly, the homozygous human B1R transgenic rat possessed a cycloheximide insensitive contractile response to DAK and DABK, with mean EC_{50} of 5.2 nM (95% confidence interval of 1.5 to 18 nM) and 810 nM (95% confidence interval of 400 to 1600 nM), respectively (Figure 5B). The nearly 160-fold selectivity of the B1R in the transgenic rat ileum for DAK relative to DABK, taken together with the slight preference of the native B1R for DABK relative to DAK, is consistent with the expected pharmacology for the human and rat receptors. The human selective compound A at concentrations of 3 and 30 nM antagonized the cycloheximide insensitive contractile response with an approximate K_b consistent with that for the human receptor (Figure 5C). The potency of compound A was very similar to its binding affinity for the human B1R, but was dissimilar to the affinity for the rat B1R (Table 2). In contrast, the cycloheximide sensitive response observed in the wild-type rat was not blocked by 30 nM compound A (Figure 5B). Taken together, these data provided compelling evidence that the human B1R expressed in the transgenic rat has the ability to elicit a functional response.

Development of an ex vivo receptor occupancy assay

The primary reason for the design and production of a human B1R transgenic rat was to develop an assay capable of ascertaining a test compound's ability to interact with the human B1R in the CNS. Administration of [35 S]compound A by either intravenous or intracerebroventricular injection did not result in the labeling within the CNS. This result was presumably due to the inability of this compound to effectively penetrate the

blood brain barrier upon intravenous injection. The compound's poor permeability properties likely result in its inability to effectively diffuse from the ventricular space into the brain parenchyma upon intracerebroventricular injection (data not shown). Therefore, compound A was not suitable for an in vivo receptor occupancy assay.

The excellent in vitro binding properties but poor tissue penetration of [³⁵S]compound A led us to explore ex vivo methods for the determination of central receptor occupancy of human selective B1R antagonists in the transgenic rats. Autoradiographic analysis of brain sections following peripheral administration of the brain penetrant antagonist compound E (*N*-[4-chloro-2-((1*R*)-1-[3'-fluoro-2'-(3-methyl-1,2,4-oxadiazol-5-yl)biphenyl-4-yl]ethyl)amino)pyridin-3-yl]-3,3,3-trifluoropropanamide) hB1 receptor binding $K_i=0.7\text{nM}$, revealed a dose dependent inhibition of [³⁵S]compound A binding (Figure 6A). While autoradiography approaches provide spatial resolution and have been used to address central receptor occupancy (Li et al., 2003; Langlois et al., 2001), these are relatively low throughput assays. The kinetics of [³⁵S]compound A binding to the human B1R suggested that a homogenate binding assay, in which the rates of association of the ligand to membranes from vehicle and hB1 antagonist treated animals were determined, could be used to measure receptor occupancy. Preliminary experiments with cerebral cortex membranes demonstrated that the rate of [³⁵S]compound A association was linear to 12 minutes after ligand addition and that the slope of the association line was directly proportional to receptor number in the incubation (data not shown). Therefore, the slope of the association line reflects free receptor and can be used to quantify receptor occupied by unlabeled drug (Figure 6B). The results from such a study with compound E were found to be comparable to those

obtained using the autoradiographic method (Figures 6C). The derived values of the dose of compound E required for 50% receptor occupancy using the homogenate and autoradiographic methods were 45ug/kg and 270ug/kg, respectively. The shift of the curve to the right with the autoradiographic method is likely due to the relatively long incubation period (90 minutes) used to obtain the results with this procedure. Therefore the ex vivo homogenate assay provides a rapid and reliable assay for determining the ability of a systemically-dosed test compound to occupy the human B1R expressed in the CNS of the transgenic rat.

Discussion:

We generated a humanized bradykinin receptor B1 rat model for the express purpose of developing an assay that enables the determination of CNS receptor occupancy of peripherally administered human specific B1R antagonists. The goal of blocking central B1R recognizes that there is a CNS site of action for this receptor, which may be important in mediating at least some of the analgesic effects of B1 antagonists (Bock et al., 2003; Couture et al., 2001; Ferreira et al., 2002; Pesquero et al., 2000). This model addresses two issues that impede the routine evaluation of the central activity of B1R antagonists; the low level of B1R expression in healthy nervous tissue and the high degree of human selectivity observed with many compounds. The model serves as a key tool in the prioritization of brain penetrant compounds for development. The homozygous human B1R transgenic rat line 4 that we developed exhibits all of the properties necessary for compound prioritization; including broad expression of the human B1R, at an adequate density throughout the brain and spinal cord, that is stable

across gender and age. Coupled with the development of a human-selective, high specific activity radioligand ($[^{35}\text{S}]$ compound A), a robust and practical ex vivo CNS receptor occupancy assay was developed.

When designing a model that would enable the development of a central B1R occupancy model, several alternative strategies to our choice of the traditional pronuclear injection technology were considered. These alternatives included viral delivery of the human B1R and the replacement of the mouse B1R with the human B1R by homologous recombination.

One advantage of using infection with a viral vector containing the gene encoding the human B1R is that it is applicable across a variety of mammalian species and thus is not confined to rodent species. However, the inability of viral particles to efficiently transverse the blood brain barrier results in the need to deliver the recombinant virus by stereotactic injection, a labor intensive procedure with relatively low throughput. The technical challenges inherent in this approach are likely to result in greater animal to animal variation than is observed with the transgenic rat. Furthermore, the size of the viral particle, coupled with natural viral tropism, results in expression of the transgene preferentially in certain cell types within a limited region. Although this may have certain advantages in the localized delivery of recombinant proteins, it is less compatible with our interest in developing a quantitative and robust measure for human B1R occupancy. Finally, the initial viral infection may induce an inflammatory response which disrupts the blood brain barrier, further complicating the evaluation of test compounds. Therefore, based on these criteria we determined that viral delivery of the

B1R was not the most effective means to develop an assay to monitor occupancy of the B1R in the CNS.

The homologous replacement of the mouse B1R with the human B1R addresses the issue of species selectivity of test compounds but does not address the low level expression of B1R in healthy tissue. One may be able to overcome the low-level of endogenous B1R expression by employing procedures that have been reported to induce the expression of the B1R, such as treatment with lipopolysaccharide (LPS) or streptozotocin (Couture et al., 2001). However, a B1R induction protocol is likely to introduce a level of complexity to the model that significantly reduces throughput by increasing animal-to-animal variability. Furthermore, the experimental paradigms that have been developed to induce the B1R in the CNS are likely to mediate disruption of the blood brain barrier (Mun-Bryce et al., 2002; Quock et al., 1988); and therefore may overestimate the ability of a compound to cross the blood-brain barrier. On the other hand, relative to our transgenic model, humanizing the mouse B1R by homologous recombination has certain advantages. Homologous recombination will result in the elimination of the endogenous rodent receptor and the faithful reproduction of the expression pattern of the native receptor. Therefore, a homologous recombination strategy may permit the determination of the efficacy of human-specific test compounds; however, for the purpose of developing a receptor occupancy measure we chose to over-express the B1R using a heterologous promoter.

Several properties of the B1R reduce the complexity in the use of this transgenic model to determine B1R receptor occupancy. Although the endogenous rat B1R is present in this model, it is transparent in most of the routine analyses that we perform.

This is due to the low level expression of the endogenous rat B1R and the human selectivity of the reagents used to assay for B1R. The inducible nature of the B1R permits one to readily discern the functional activity that arises from the human B1R in the isolated ileum, as cycloheximide treatment blocks the synthesis of the rat B1R.

There are properties of this transgenic model that limit its use, particularly when considering its potential for testing the efficacy of human selective compounds. Since the human B1R is functional, it should be possible to elicit a response that could be utilized to assess B1R antagonists. However, we expect that many of the experimental paradigms that have been developed to study B1R function *in vivo* will result in the induction of the native rat B1R. In addition, the use of the NSE promoter to control the human B1R expression results in constitutive expression, as is demonstrated in the ileum contraction experiment. Thus the transcription of the transgenic B1R will not respond to tissue injury in the same manner as the native rat B1R. Nevertheless, it remains possible that this transgenic model could be exploited to study molecular systems acting downstream of transcriptional regulation that modulate B1R activity in response to tissue damage. With respect to testing the efficacy of human selective compounds, however, the presence of both the human and rat B1Rs in an inflammatory setting in this transgenic rat severely complicates this analysis. Therefore, the transgenic rat model that we describe is not appropriate for testing human selective B1R antagonists using assay conditions in which the endogenous rat B1R is induced, e.g. LPS or formalin treatment.

The B1 receptor exhibits a relatively high level of basal signaling and constitutively active mutants that enhance this basal activity have been identified (Leeb-Lundberg et al., 2001). These data suggest the possibility that over-expression of the

B1R will result in ligand independent activity. The demonstrated functionality of the human B1R in the transgenic rat coupled with this potential for ligand independent activity raises the possibility of developing a behavioral assay to monitor the in vivo effectiveness of human selective B1R antagonists. The requirements for such a behavior response are that it is dependent upon the ectopic expression of the transgene, and that the endogenous rat B1R does not participate. Chao and colleagues recently reported a transgenic mouse in which they used the CMV promoter to over-express either the wild type or a constitutively active mutant rat B1R (Ni et al., 2003). Phenotypic analysis of the hemodynamic properties of this transgenic mouse did not reveal any activity attributable to either elevated wild type basal activity or constitutive activity of the mutant receptor. Surprisingly, this transgenic mouse responded to intravenous administration of the B1 agonist des-Arg⁹BK with an increase in mean arterial blood pressure, a response that is not observed in nontransgenic control mice (Ni et al., 2003). In contrast, administration of a B1 agonist, following induction of the endogenous B1R by an agent such as LPS, has been previously found to decrease blood pressure (Marceau et al., 1998). Therefore, the aberrant response observed in the CMV rat B1R transgenic mouse may be a consequence of ectopic expression of B1R. To date, we have not identified a particular phenotype or physiological response to B1 activation in the NSE human B1R transgenic rat that is acquired as a consequence of expression of the human B1R. In preliminary studies, the following endpoints were compared between wild-type and heterozygous transgenic rats where no difference between genotypes were observed (unpublished observations) with or without intracerebroventricular injection of DAK (1µg and 10 µg; n=5/treatment group per gender): hot plate reaction time (53°C or

55°C), motor performance on variable speed rotarod, hindpaw withdrawal threshold to mechanical stimulus, and core body temperature. In addition, there was no effect of the transgene on exploratory locomotor activity within the first 30 minutes of placement into a novel environment. Therefore, based on this initial exploration, no phenotype emerged that had the potential to be exploited in the analysis of the activity of human selective B1R antagonists.

In conclusion, we have developed a humanized B1R transgenic rat with an expression level for the B1R in the brain and spinal cord that was sufficient to develop robust assays for the determination of a test compounds ability to occupy B1R. This receptor occupancy determination provides critical information in the selection of B1R antagonists that have the greatest potential to become therapeutic agents.

Acknowledgments:

We thank Drs. Tony Ford-Hutchinson and Jeff Conn for support and encouragement.

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Footnotes:

- a) This work was presented in abstract form at the 2003 Annual Meeting of the Society for Neuroscience in New Orleans, LA.
- b) To whom reprint requests should be addressed: J. Fred Hess, Merck Research Lab, WP26A-3000, P.O. Box 4, West Point PA 19486

Figure Legends:

Figure 1. (A) Schematic of the construct utilized to produce the humanized B1R transgenic rat. The construct contains the rat neuron specific enolase promoter, CMV intron A, the human B1 receptor coding sequence and the bovine growth hormone polyadenylation signal. (B) Autoradiogram of Southern blot analysis of rat genomic DNA derived from independent founders resulting from the pronuclear injection of the NSE/B1 construct. Equivalent amounts, 10 ug, of *Eco* RI digested genomic DNA were fractionated on a 1% agarose gel and Southern blotted with a transgene specific probe. Transgenic positives are lane 1 (founder number 4), 3 (founder 12), 4 (founder 14), 5 (founder 15), and 7 (founder 23), lanes 2 and 6 are rats that are negative for the transgene. Lanes 8 and 9 correspond to a spiked control corresponding to 1 and 10 copies, respectively, of the transgene per genome equivalent. (C) Autoradiogram of Southern blot analysis of *Dra* I digested rat genomic DNA probed with a fragment immediately upstream of the transgene insertion site. Lane 1: wild type; 2: heterozygous; 3: homozygous transgenic.

Figure 2. (A) Saturation binding of membranes isolated from whole brain homogenates prepared from line 4 human B1R transgenic rats. Total binding resulting from increasing concentrations of [³H]DALK (■), nonspecific binding determined by competition with cold DALK (▲), and specific binding (▼). Scatchard plot (inset). (B) [³H]DALK binding with membranes isolated from whole brain homogenates prepared from line 4 human B1R transgenic rats. Competition with compound C 2-[(2R)-1-[(3,4-

dichlorophenyl)sulfonyl]-3-oxo-1,2,3,4-tetrahydroquinoxalin-2-yl}-*N*-{2-[4-(4,5-dihydro-1*H*-imidazol-2-yl)phenyl]ethyl}acetamide (■), and compound D 3-[[2,2-difluorocyclopropyl)carbonyl]amino}-*N*-{(1*R*)-1-[2'-(methoxycarbonyl)-5'-methylbiphenyl-4-yl]ethyl}-4-methylpyridin-2-aminium chloride (◆).

Figure 3. Autoradiography using [³⁵S]compound A of brain section isolated from individual animals from transgenic rat lines 4, 14, 15 and wild type. Total binding was obtained by incubation of a 20 μM coronal section in the presence of 10 pM [³⁵S]compound A (left-hand side) and nonspecific binding was determined with the addition of cold 500 nM compound B (right-hand side). The slices were exposed to film and then digitized using the MCID M5 Software.

Figure 4. Autoradiogram of coronal brain and spinal cord sections isolated from human B1R line 4 heterozygous transgenic rat. Total binding was determined with 0.3 nM [³H] DALK and nonspecific binding was determined by adding 200 nM of compound B. Slices are ordered anterior to posterior. There is no detectable binding of [³H]DALK in non-transgenic rats. Abbreviations: BST (bed nucleus of the stria terminalis), CPu (caudate putamen), DpG (deep gray layer of the superior colliculus), InG (intermediate gray layer of the superior colliculus), Op (optic layer of the superior colliculus).

Figure 5. Functional response of rat B1R and human B1R in wild type (A, B) and transgenic (C) rats. Contraction of isolated ileum in a tissue bath was stimulated by the addition of increasing concentrations of DAK and is expressed relative to the contraction in response to 40 mM KCl. (A) The native rat B1R responds to DAK with an EC₅₀ of 6.4

nM (■). This response is abolished in the presence of 70 μ M cycloheximide (▲). (B) In the absence of cycloheximide the response to DAK in the wild type rat (vehicle ■, n = 4) is not blocked by 30 nM (▲, n = 4) compound A. (C) In the presence of 70 μ M cycloheximide the B1R present in the transgenic rat responds to DAK with an EC₅₀ of 5.2 nM (vehicle ■, n = 6). This response is blocked by 3 nM (▲, n = 6) and 30 nM (●, n = 4) of the human B1R selective antagonist compound A, with an approximate K_b of 0.27 nM.

Figure 6. Dose dependent inhibition of [³⁵S]compound A labeling of human B1R by Compound E. Animals received either vehicle or the indicated intravenous dose of Compound E and were sacrificed thirty minutes post dosing. The brain was bisected, with ½ being used for autoradiography and the second ½ for tissue homogenate binding. (A) Twenty micron coronal brain sections were incubated with 10 pM [³⁵S] compound A for 90 min, washed, exposed to film, and subsequently evaluated with the MCID M5 software package. (B) Linear regression of [³⁵S]compound A association to brain membranes prepared from transgenic rats. Groups of two rats were dosed intravenously with vehicle (▼), or 0.024 (■), 0.12 (●), 0.60 (Δ), 3.0 (▽), 15.0 (◇) mg/kg Compound E. Membranes were prepared and [³⁵S]compound A association performed. (C) Comparison of compound E ex vivo occupancy results using either autoradiographic (▲) or homogenate binding (■) methods. The striatal optical densities from 6A were used for the calculation of the autoradiographic receptor occupancies while the homogenate results were derived from the data in 6B. The same animals were used for both analyses.

Table 1 Expression Level of B1R in the CNS of Transgenic Rat Lines

Saturation binding analysis using either [³H]DALK or [³⁵S] compound A was performed on membrane protein homogenates prepared from either whole brain or spinal cord. Het is heterozygous, Hom is homozygous. Data is presented mean ± standard error.

Line	B1R (fmole/mg prot)	KD DALK (nM)
4 Het	39 ± 4.2 (n=18)	0.17 ± 0.043
4 Hom	84 ± 8.0 (n=9)	0.18 ± 0.032
14	4 (n=1)	0.24
15	7 (n=1)	0.19
23	8.1 ± 5.9 (n=3)	0.31 ± .079

Table 2 Pharmacological profile of B1R in transgenic rat line 4 compared to recombinant human and rat B1R expressed in CHO cells

	DALK	Compound A	Compound B	Compound C	Compound D
Line 4^a	0.25 ± 0.032	0.018 ± 0.0018	0.066 ± .032	0.027 ± .0026	0.92 ± .088
Human B1 CHO^a	0.19 ± 0.013	0.014 ± .0048	0.073 ± .010	0.014 ± .0043	0.85 ± .082
Rat B1 CHO^a	18 ± 9.8	34 ± 8.1	0.37 ± 0.029	250 ± 160	5500
Wild type ileum^b	ND^c	>30	1.9	800	ND
Line 4 ileum^b	ND	0.27	0.27	0.032	ND

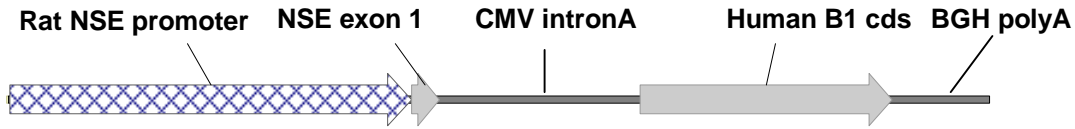
(a)K_i, in nM, was determined by competition binding analysis with radiolabeled [³⁵S]compound A. Values were determined using membranes prepared from whole brain homogenates of Line 4 human B1R transgenic rats, membranes from recombinant human B1R expressed in CHO cells, or membranes from recombinant rat B1R expressed in CHO cells. The mean of three to five independent determinations ± standard error was determined for all values except for rat B1 CHO compound D, n = 1.

(b)K_b, in nM, was approximated based on the decrease in EC₅₀ of des-Arg¹⁰kallidin induced contraction of the ileum resulting from the presence of the antagonist.

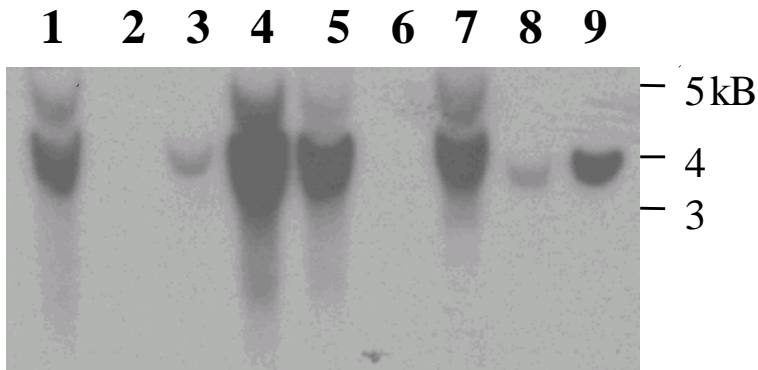
(c)ND is not determined

Figure 1

A



B



C

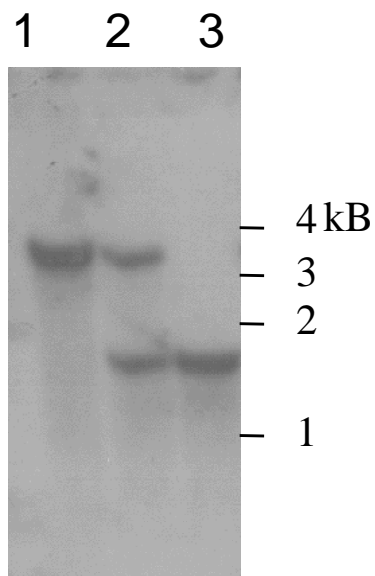
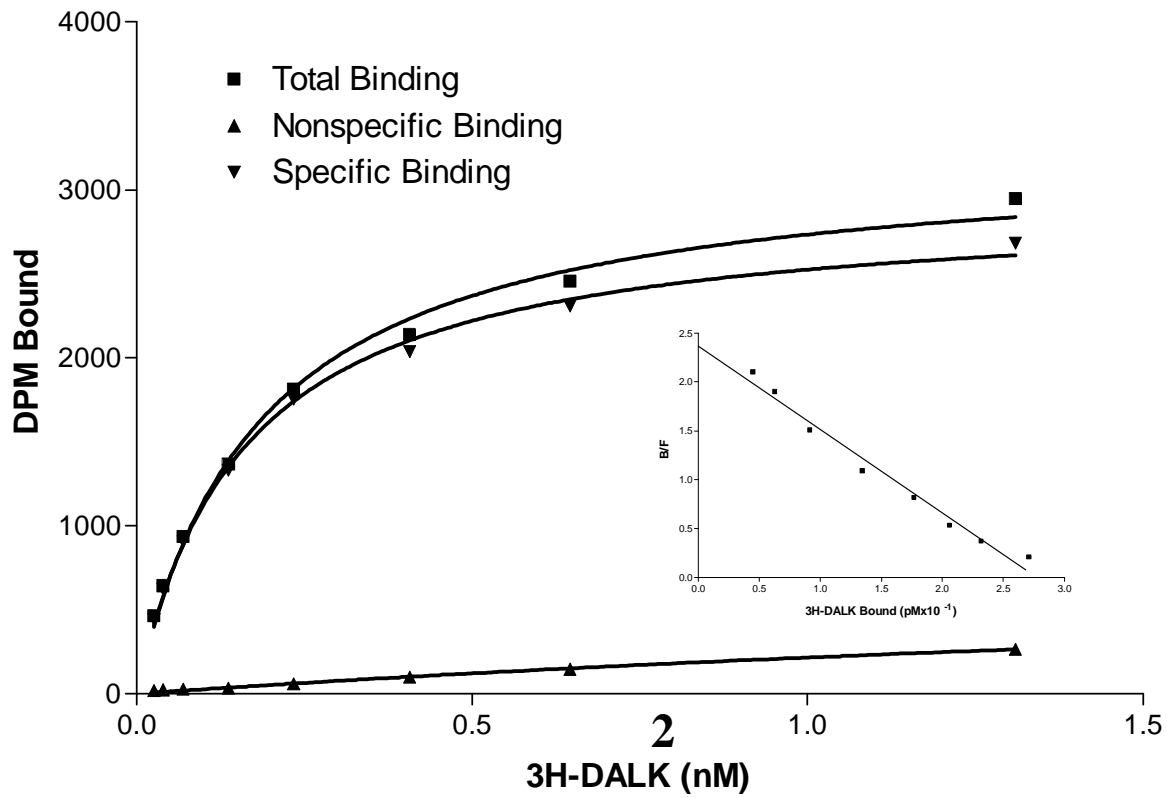


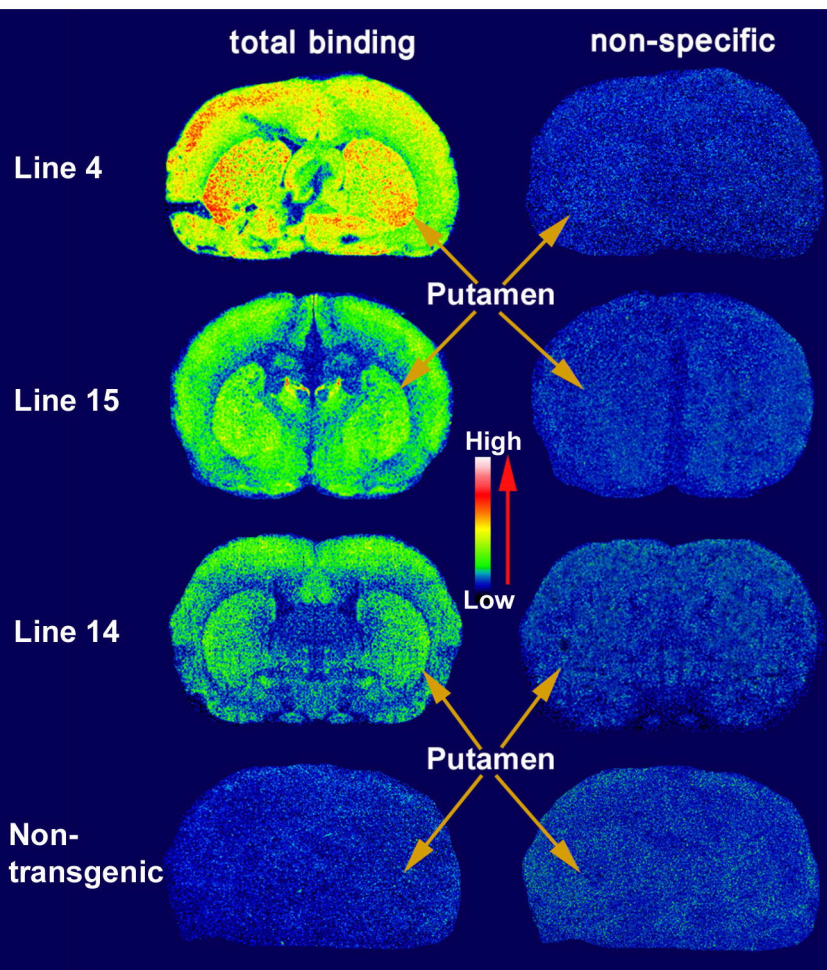
Figure 2

A



B





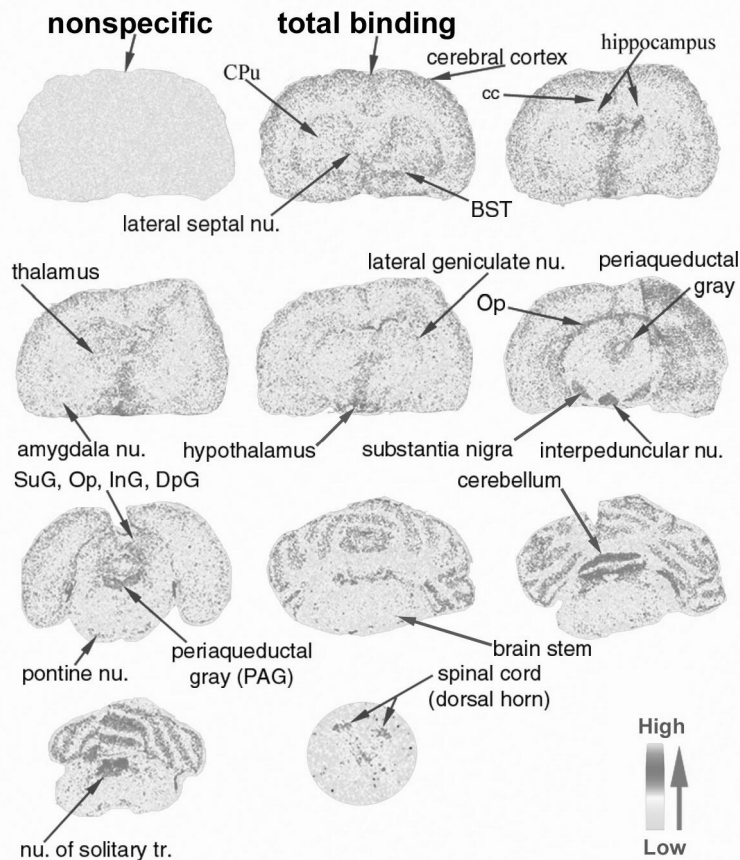


Figure 5

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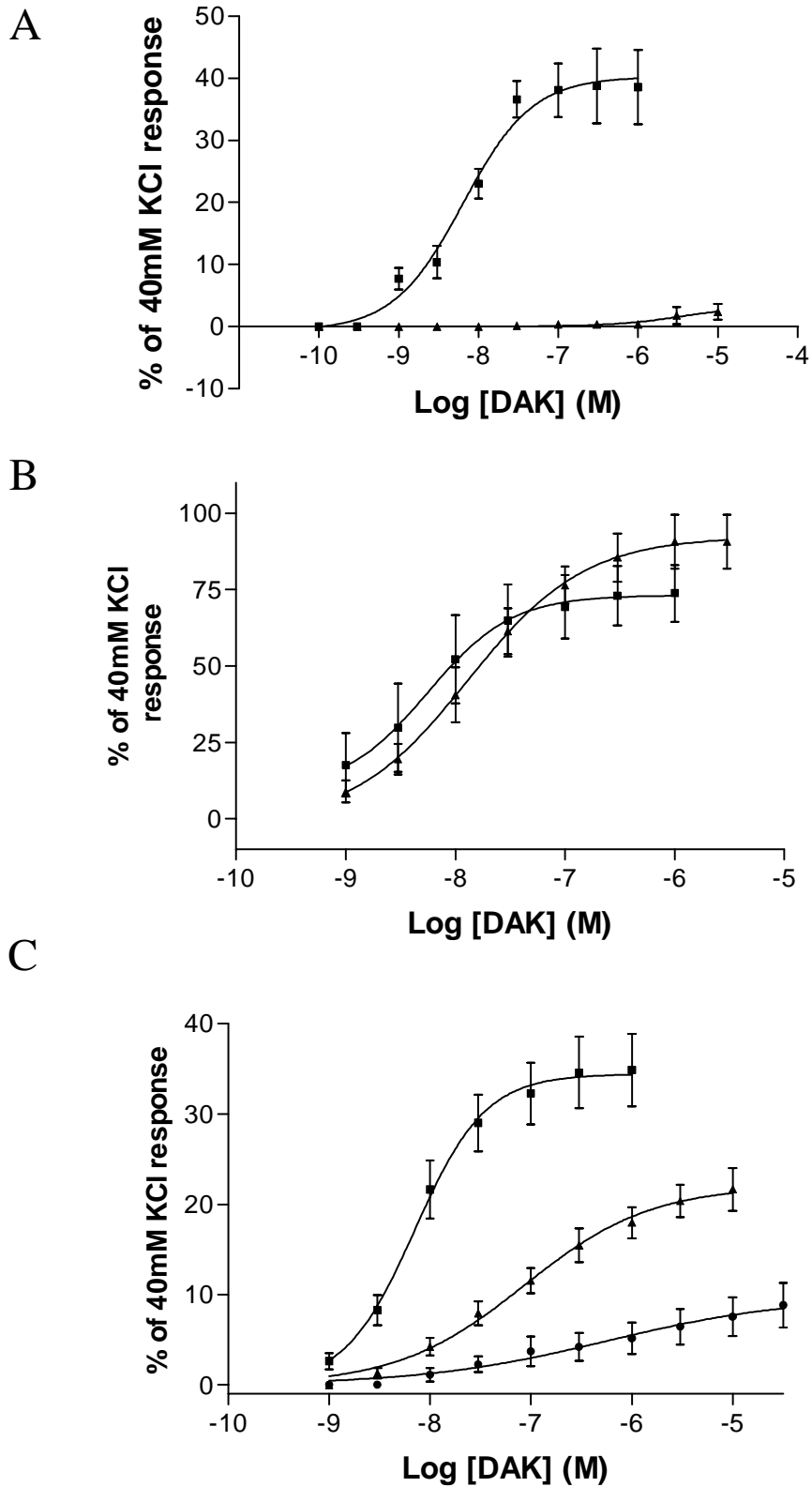


Figure 6A

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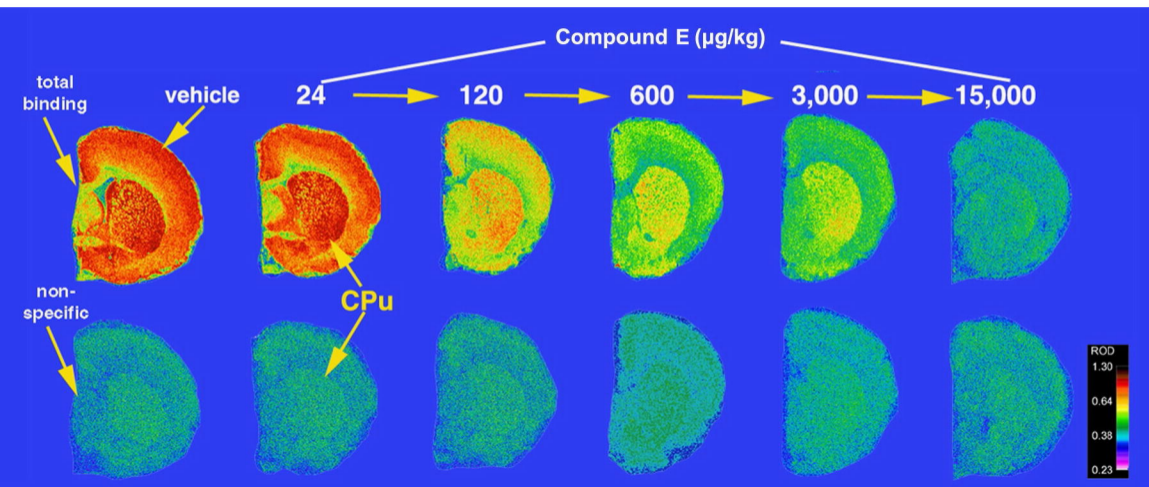
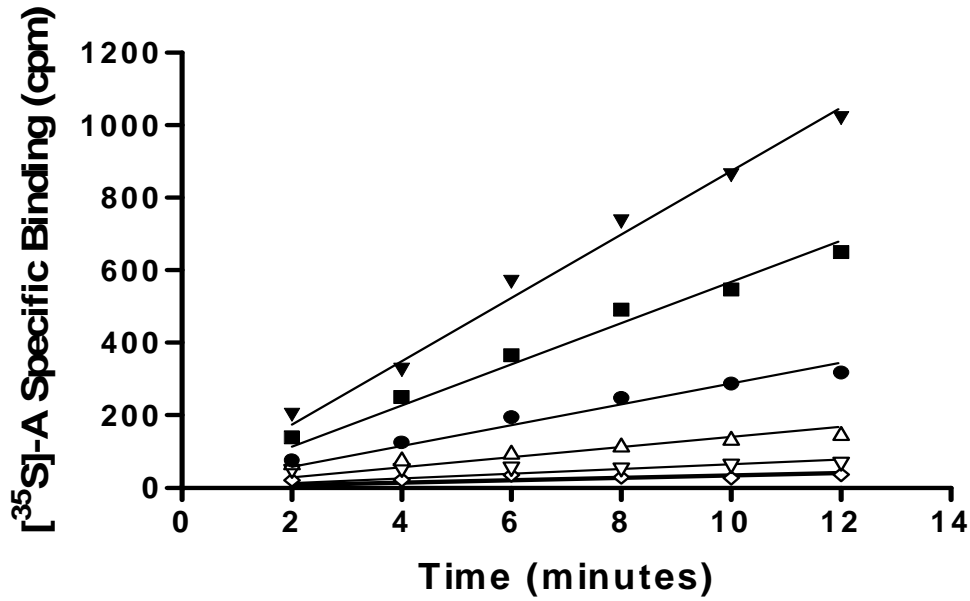


Figure 6

B



C

