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Human locus coeruleus neurons express the $GABA_A$ receptor γ2 subunit gene and produce benzodiazepine binding

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ABSTRACT

Noradrenergic neurons of the locus coeruleus project throughout the cerebral cortex and multiple subcortical structures. Alterations in the locus coeruleus firing are associated with vigilance states and with fear and anxiety disorders. Brain ionotropic type A receptors for γ-aminobutyric acid (GABA) serve as targets for anxiolytic and sedative drugs, and play an essential regulatory role in the locus coeruleus. $GABA_A$ receptors are composed of a variable array of subunits forming heteropentameric chloride channels with different pharmacological properties. The γ2 subunit is essential for the formation of the binding site for benzodiazepines, allosteric modulators of $GABA_A$ receptors that are clinically often used as sedatives/hypnotics and anxiolytics. There are contradictory reports in regard to the γ2 subunit's expression and participation in the functional $GABA_A$ receptors in the mammalian locus coeruleus. We report here that the γ2 subunit is transcribed and participates in the assembly of functional $GABA_A$ receptors in the tyrosine hydroxylase-positive neuromelanin-containing neurons within postmortem human locus coeruleus as demonstrated by *in situ* hybridization with specific γ2 subunit oligonucleotides and autoradiographic assay for flumazenil-sensitive [^{3}H]Ro 15-4513 binding to benzodiazepine sites. These sites were also sensitive to the α1 subunit-preferring agonist zolpidem. Our data suggest a species difference in the expression profiles of the α1 and γ2 subunits in the locus coeruleus, with the sedation-related benzodiazepine sites being more important in man than rodents. This may explain the repeated failures in the transition of novel drugs with a promising neuropharmacological profile in rodents to human clinical usage, due to intolerable sedative effects.

The locus coeruleus (LC) is the major source of noradrenaline-containing neurons in the brain. It is ventrolateral to the fourth ventricle in the pons. Increased firing of the LC is associated with a state of increased vigilance, stress exposure, fear, and anxiety. Subjective sensations of anxiety and physical symptoms associated with panic disorder can be elicited in healthy human subjects following noradrenaline administration (reviewed in [6,7]). Insomnia is frequently associated with stress and anxiety. The LC has been implicated as a component of the neural network regulating sleep and wakefulness, being active during the awake state and hence known as an arousal centre (reviewed in [27]). Hence, inhibition of the LC neurons might induce strong anxiolytic/sedative/hypnotic effects.

The major GABAergic input source to the core of the LC is the nucleus prepositus hypoglossi, inhibiting the LC firing by activating presumably both pre- and postsynaptic $GABA_A$ receptors [12,15,26]. $GABA_A$ receptors are heteropentameric ligand-gated anion channels, composed of variable combinations of α1–6, β1–3, γ1–3, δ, ε, θ and π subunits, forming functionally diverse receptors. These receptors differ in their channel kinetics, rate of desensitization and affinity for GABA and allosteric modulators (reviewed in [23]). The rare subunits ε and θ are highly enriched in the rodent and primate LC [5,24,25,29]. The unique repertoire of $GABA_A$ receptor subunits in a specific neuronal network regulating particular brain functions could provide an opportunity to develop subunit-selective drugs acting on selected neuronal populations.

Benzodiazepines are generally prescribed as anxiolytic and sedative–hypnotic drugs. The $GABA_A$ receptor γ subunit, in particular the γ2 variant, is essential for benzodiazepine binding and efficacy [18]. Some studies have suggested the presence of γ2 subunit mRNA expression in the LC region of rats [8,9], not showing the specific expression in the noradrenaline neurons. Fritschy and Mohler [13] have shown an immunohistochemical signal for γ2 subunit in the rat LC. However, many rodent studies do not find the

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Table 1
Demographic data of subjects.

Subject (ID)	Sex	Age (years)	PMI[a] (h)	Cause of death	Toxicology	Diagnoses
1228	Male	49	50.5	Natural	NDD[b]	Negative
1548	Male	37	26	Natural	NDD	Negative
1550	Male	20	32	Natural	Ethanol	Negative
1558	Female	23	17.5	Natural	NDD	Negative
1563	Female	21	18	Natural	Diphenhydramine	Negative

[a] Postmortem interval.
[b] No drugs detected.

γ2 subunit gene expression in the LC (e.g., [2,11,20]), suggesting that LC is insensitive to benzodiazepines. Accordingly, the LC would not be implicated in the direct mediation of anxiolysis or sedation by these drugs. However, there might be considerable species differences in the effects of benzodiazepines in the LC. The present study was aimed to investigate the expression of $GABA_A$ receptor γ2 subunit and benzodiazepine binding ability in the human LC. After this study was submitted, Waldvogel et al. [28] have demonstrated γ2 subunit immunoreactivity in the pigmented neurons of human LC.

Postmortem brain material for normal human brain was obtained from the brain tissue collection of the Section on Neuropathology of the Clinical Brain Disorders Branch, Genes Cognition and Psychosis Program of the Intramural Research Program of the National Institute of Mental Health under a protocol approved by the IRB of the NIMH, with informed consent of the next of kin. The collection, screening, and analysis of the subjects used in this study have been previously described by Lipska et al. [16]. These samples of LC and cerebellar cortex (2 females, 3 males, average age 30 ± 12.6 years) were used for immunohistochemical, *in situ* hybridization and ligand autoradiographic procedures.

The cause of death was determined by a pathologist at autopsy and the toxicology data were obtained on each case with an assay of either brain or blood, depending upon availability (Table 1). The interval between death and tissue collection ranged from 16 to 50.5 h. Immediately after autopsy, the pons and cerebellar cortex were cut into 2–3 cm thick slabs, flash frozen in a slurry of dry ice and isopentane, and kept at −80 °C. Fourteen-μm-thick sections were cut from the slabs of pons and cerebellar cortex with a Leica CM 3050S cryostat (Leica Microsystems, Benheim, Germany) at −20 °C for the histochemical experiments. The sections were thaw-mounted onto gelatin-coated (for immunohistochemistry and ligand autoradiography) or SuperFrost (for *in situ* hybridization histochemistry) object classes (Menzel-Gläser) and stored frozen at −70 °C.

Tyrosine hydroxylase (*TH*) *immunostaining* was performed to localize the LC on the sections, and adjacent sections were used for *in situ* hybridization and ligand autoradiography. Sections were fixed in cold acetone for 5 min and washed briefly in 1× phosphate-buffered saline (PBS), pH 7.4. Then the sections were incubated at room temperature with the 1:25 dilution of primary rabbit anti-TH affinity-purified polyclonal antibody (AB152; Chemicon International, Temecula, USA), the 1:50 dilution of secondary biotinylated anti-rabbit goat IgG (PK-6105; Vectastain Elite ABC Kit, Vector laboratories) and with the avidin/biotinylated horseradish peroxidase enzyme complex ABC (Vectastain Elite ABC Kit, Vector laboratories) for 4 min each and briefly rinsed with 1× PBS between each step. After the color development with diaminobenzidine (DAB) (Vector laboratories) for 60 s, the sections were rinsed in H_2O, and then dehydrated in 70%, 95% and 100% ethanol (30 s each) and xylene (twice for 5 min each). Finally, glass cover slips (Menzel-Gläser) were mounted on the sections by gently laying them on to a drop of mounting medium. Negative controls were carried out in the absence of the primary antibody. The LC sections were scanned (Epson expression 1680 Pro) with digital manipulation (shadow 179, gamma 2.89, highlight 215) in order to sharply elicit the TH-positive cells that were clearly observable with a light microscope.

For in situ hybridization experiments, the air-dried LC-containing and cerebellar cortical sections from one subject (ID: 1550) were fixed in ice-cold 4% paraformaldehyde for 5 min. The sections were washed in 1× PBS at room temperature for 5 min, dehydrated in 70% ethanol for 5 min and stored in 95% ethanol at 4 °C until used. Two different antisense DNA oligonucleotide probes (45-mers) complementary to the human $GABA_A$ receptor γ2 subunit mRNA sequence (nucleotides 1442–1486 and 1695–1739; GenBank accession number NM_198904) and analogous sense probes were synthesized (Oligomer Oy, Helsinki, Finland). Both antisense probes displayed 100% query coverage with transcript variants 1, 2 and 3 (GenBank accession numbers NM_198904.1, NM_000816.2 and NM_198903.1, respectively). Poly[^{35}S]dA ([^{35}S]dATP from PerkinElmer Life and Analytical Sciences, Boston, MA, USA) tails were added to the 3′-ends of the probes by deoxynucleotidyl transferase (Promega Corporation, Madison, WI, USA). Unincorporated nucleotides were removed by Illustra ProbeQuant G-50 Micro Columns (Amersham Biosciences, Buckinghamshire, UK). The labelling efficiency (200 000–330 000 cpm/μl) was determined by a scintillation counter. The labelled probe was diluted to 0.06 fmol/μl with hybridization buffer (containing 50% formamide, 10% dextran sulphate, 4× SSC). Nonspecific controls for the antisense probes were produced by adding 100-fold excess of unlabelled probes. The hybridization occurred under glass cover slips (Menzel-Gläser) over-night at 42 °C. Finally, the slides were washed in 1× SSC at room temperature for 10 min, in 1× SSC at 55 °C for 30 min, and 1× SSC, 0.1× SSC, 70% EtOH and 95% EtOH at room temperature for 1 min each. The sections were then air-dried and exposed to BioMax MR film (Eastman Kodak Company, Rochester, NY, USA) for up to a week. The films were scanned for images (Epson expression 1680 Pro). The sections were then processed for emulsion autoradiography, by dipping them in Kodak autoradiography emulsion (Eastman Kodak Company, Rochester, NY, USA) diluted in 600 mM ammonium acetate (1:1 volume) in the 42 °C water bath, air-dried over-night in the dark and exposed in a light-tight slide box at 4 °C for 6 weeks. Thereafter, the sections were developed at 15 °C in Kodak D-19 Developer for 3 min, water for 30 s, and Kodak fixer for 5 min. The sections were then washed twice in water for 5 min and air-dried over-night at room temperature. The dry sections were counterstained by 0.125% thionin, rinsed with water, dehydrated in ethanol series (70%, 95%, 100%) and xylene. At the end, the cover slips were mounted on the sections. Conventional transmitted and darkfield images were acquired using Olympus AX70 microscope with UPlanF1 20×/0.50 NA or PlanApo 60×/1.40 NA Oil objectives.

[^{3}H]Ro 15-4513 autoradiographic binding assay (modified from [21]) was performed to label the benzodiazepine binding sites and to assess the zolpidem sensitivity of the receptors. Brain sections from five subjects (IDs: 1228, 1548, 1550, 1558 and 1563) were pre-incubated in ice-cold 50 mM Tris–HCl buffer, pH 7.4, containing 120 mM NaCl for 15 min. The final incubation was performed in the pre-incubation buffer containing 15 nM (160 cpm/μl) [^{3}H]Ro 15-4513 (PerkinElmer Life and Analytical Sciences, Boston, MA,