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Neurochemistry of the afferents to the rat cochlear root nucleus: Possible synaptic modulation of the acoustic startle

R Gómez-Nieto^{1,2,▲}, JAC Horta-Junior^{1,3,▲}, O Castellano¹, MJ Herrero-Turrión¹, ME Rubio², and DE López^{1,*}

¹Instituto de Neurociencias de Castilla y León, Universidad de Salamanca, 37007 Salamanca, Spain

²Department of Physiology and Neurobiology, University of Connecticut, Storrs, Connecticut 06269-3156, USA

³Departamento de Anatomia, Instituto de Biociências, Universidade Estadual Paulista, Botucatu, São Paulo, 18618-000, Brazil

Abstract

Afferents to the primary startle circuit are essential for the elicitation and modulation of the acoustic startle reflex (ASR). In the rat, cochlear root neurons (CRNs) comprise the first component of the acoustic startle circuit and play a crucial role in mediating the ASR. Nevertheless, the neurochemical pattern of their afferents remains unclear. To determine the distribution of excitatory and inhibitory inputs, we used confocal microscopy to analyze the immunostaining for vesicular glutamate and GABA transporter proteins (VGLUT1 and VGAT) on retrogradely labeled CRNs. We also used reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemistry to detect and localize specific neurotransmitter receptor subunits in the cochlear root. Our results show differential distributions of VGLUT1- and VGAT-immunoreactive endings around cell bodies and dendrites. The RT-PCR data showed a positive band for several ionotropic glutamate receptor subunits, M1-M5 muscarinic receptor subtypes, the glycine receptor $\alpha 1$ subunit (GlyR $\alpha 1$), GABA_A, GABA_B, and subunits of $\alpha 2$ and β -noradrenergic receptors. By immunohistochemistry, we confirmed that CRN cell bodies exhibit positive immunoreaction for the GluR3 and NR1 glutamate receptor subunits. Cell bodies and dendrites were also positive for M2 and M4, and GlyR $\alpha 1$. Other subunits, such as GluR1 and GluR4 of the AMPA glutamate receptors, were observed in glial cells neighboring unlabeled CRN cell bodies. We further confirmed the existence of noradrenergic afferents onto CRNs from the locus coeruleus by combining tyrosine hydroxylase immunohistochemistry and tract-tracing experiments. Our results provide valuable information toward understanding how CRNs might integrate excitatory and inhibitory inputs, and hence how they could elicit and modulate the acoustic startle reflex.

*Correspondence to: Dr. Dolores E. López, Laboratorio de Neurobiología de la Audición, Instituto de Neurociencias de Castilla y León, Universidad de Salamanca, Alfonso X El Sabio s/n, 37007 Salamanca, Spain. Phone: (34)923294563; FAX: (34)923294549. E-mail: lopezde@usal.es.

▲These authors contributed equally to this work

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Keywords

neurotransmitter receptors; receptor expression; VGAT; VGLUT; locus coeruleus; cochlear root neurons

Introduction

Cochlear root neurons (CRNs) are interspersed among the fibers of the central portion of the cochlear nerve and play a crucial role in eliciting the acoustic startle reflex (ASR) in rats (Lee et al., 1996; Yeomans and Frankland, 1996; Koch and Schnitzler, 1997; López et al., 1999). The ASR may be modified quantitatively or qualitatively by a range of natural and experimental conditions (Swerdlow et al., 2000; Bell et al., 2003), a feature that reflects the plasticity of this reflex. The ASR can be diminished by habituation (Gonzalez-Lima et al., 1989; Pilz and Schnitzler, 1996), a previous stimulus (Hoffman and Fleshler, 1963; Braff et al., 2001; Fendt et al., 2001; Swerdlow et al., 2007), positive stimuli (Koch, 1999), and by administration of certain drugs (Swerdlow and Geyer, 1993; Bakshi et al., 1994; Geyer et al., 2001). Conversely, the ASR may also be increased during situations of fear potentiation (Anisman et al., 2000; Davis, 2006; Winslow et al., 2007), anxiety (Kaviani et al., 2004; Prehn et al., 2006), and stress (Andreski et al., 1998; Stam, 2007).

The mechanisms responsible for ASR modulation are likely to influence the basic circuit of the ASR, which in the rat is composed of ganglion cells of the organ of Corti, CRNs, reticulospinal neurons of the caudal pontine reticular nucleus (PnC), and motoneurons of the spinal cord (Lee et al., 1996; Yeomans and Frankland, 1996; Koch and Schnitzler, 1997; López et al., 1999; Nodal and López, 2003). Much is known about the integrative role that the PnC plays as the principal center of stimulus convergence in the ASR circuit (Koch et al., 1992; Lingenhöhl and Friauf, 1994; Lee et al., 1996; Fendt and Koch, 1999; Fendt et al., 2001; Zhao and Davis, 2004); however, the role of CRNs in ASR modulation remains to be fully defined because the nature of their neural inputs is not well known.

The main source of input to CRNs stems from collaterals of auditory nerve afferents originating in the cochlea (Harrison et al., 1962; Osen et al., 1991; Berglund et al., 1996). Nevertheless, CRNs are innervated by at least three more types of boutons with different ultrastructural characteristics (Merchán et al., 1988; Gómez-Nieto et al., 2008). Afferents from the cochlea are assumed to be glutamatergic (Furness and Lawton, 2003), as is observed in other auditory regions such as the cochlear nucleus (Rubio, 2006; Zhou et al., 2007). In addition, many reports have described cholinergic afferents to the cochlear root area (Vetter et al., 1993; Yao and Godfrey, 1999) arising from the ventral nucleus of the trapezoid body (VNTB) (Gómez-Nieto et al., 2008). Currently, their role remains uncertain, largely due to a lack of information about the receptor types expressed by CRNs.

Osen et al. (1991) described inhibitory inputs on CRNs with small gamma-aminobutyric acid (GABA)-immunoreactive terminals distributed mainly on primary dendrites, where small glycine-like terminals were also observed, but in lower numbers. Scarce GABA and glycine-immunoreactive boutons on the somata were also described. An effective inhibition of CRNs may cause changes in their response, which would in turn be reflected in alterations of the elicited startle reflex, ranging from a slight decrease in the ASR, with little or no effect on neural response latency, to a complete elimination of the reflex. Accordingly, inhibitory inputs may play a modulatory role in the startle reflex that decreases the response amplitude, such as in prepulse inhibition (PPI) (Hoffman and Searle, 1965).

Another important neurotransmitter that modulates the ASR is noradrenaline (Davis et al., 1977; Astrachan et al., 1983; Saitoh et al., 1986; Alsene et al., 2006). The locus coeruleus (LC) provides noradrenergic inputs to cochlear nuclei (Klepper and Herbert, 1991; Ebert, 1996; Thompson, 2003), the superior olivary complex (Mulders and Robertson, 2001), and the inferior colliculus (Klepper and Herbert, 1991); however, the presence of noradrenergic afferents to the CRNs has not been investigated, in spite of the CRNs essential role in eliciting the startle reflex (Lee et al., 1996).

To study CRN afferents, we performed a multi-technical approach, including tract-tracing and immunohistochemical localization of neurotransmitter transporters and receptors, as well as an evaluation of the expression of the main receptor subtypes within the cochlear root. Based on current anatomical (Merchán et al., 1988; Osen et al., 1991; Gómez-Nieto et al., 2008) and physiological (Sinex et al., 2001) data, we focused the analysis of receptor expression on those related to fast excitatory transmission ionotropic glutamate receptors (iGluR), GABA receptors (GABA_A and GABA_B and GABA_C), muscarinic acetylcholine receptors (M1-5 subtypes), the glycinergic receptor ($\alpha 1$), and noradrenergic receptors ($\alpha 2A-C$ and $\beta 1-3$) in the cochlear root.

The goal of the experiments reported here was to determine the neurochemical environment of CRNs with a view to understanding their activity and the consequent elicitation and modulation of the auditory startle reflex.

Experimental Procedures

Animals and experimental design

Twenty-nine adult Wistar rats weighing 290-320 g were used. The experiments were conducted according to the guidelines for the use and care of laboratory animals of the European Communities Council Directive (DOCE L 222; 24-08-1999) and with those established by the United States (NIH publication No. 80-23). The animals were housed in groups of three prior to surgery and individually thereafter. Throughout the study period, all animals had free access to water and laboratory chow.

To study the topographical distribution and the neurochemistry of the inputs onto CRNs, we retrogradely labeled their somata and dendrites by injecting fluorescein isothiocyanate-conjugated dextran (D-FITC) into the trapezoid body (tz), and detected the vesicular glutamate transporter subtype 1 (VGLUT1) and the vesicular GABA transporter (VGAT) by immunohistochemistry.

In addition, several RT-PCRs were performed in an attempt to screen for the presence of different receptor mRNA expressions, and determine their relative quantity. On the basis of genetic expression, a separate design was used to immunohistochemically study the startle reflex related neurotransmitter receptors in the cochlear nerve root. Six rats were used, and from each animal, two bin series were used per subunit receptor. Furthermore, tyrosine hydroxylase (TH) immunohistochemistry and the injection of biotinylated dextran amine (BDA) into the locus coeruleus to study noradrenergic fibers were prompted by the observation of noradrenergic receptors in the cochlear root.

Injection of neuronal tracers

Neuronal tracers were injected after deep anesthetization of the animal through intramuscular administration of a mixture of xylazine (7 mg/kg) and ketamine (40 mg/kg). When necessary, additional anesthesia (one-fifth of the initial dose) was given during surgery. Each animal was placed in a stereotaxic frame (Kopf, Tujunga, CA, USA) using the head-positioning procedure

described by Paxinos and Watson (2005). The skull was exposed by a midline scalp incision, and a craniotomy was made over the injection coordinates.

Four animals received an injection of D-FITC (10,000 MW; #D-1820; Molecular Probes Inc., Eugene, OR, USA) in the tz to retrogradely label the CRNs. A volume of 0.10 - 0.20 μ l of D-FITC (10% in distilled water) was pressure-injected using a Hamilton syringe (#710, Hamilton Co, Reno, Nevada, USA) attached to a Stereotaxic Injector (#53311, Stoelting Co, Wood Dale, IL, USA). The coordinates to target the extreme superficial portion of the tz, where CRN axons course to innervate the overlying reticular formation (López et al., 1999) were obtained from the atlas of the rat brain of Paxinos and Watson (2005).

Five animals were used for tract-tracing CRN afferents from the locus coeruleus (LC). These animals received an injection of biotinylated dextran amine (BDA, 10,000 MW, #D-1956; Molecular Probes) into the left LC following stereotaxic coordinates previously described (Fritschy and Grzanna, 1990). Since the LC is topographically organized, the injection sites were preferentially located in and around the ventral half of the nucleus, where the descending projections originate (Guyenet, 1980, Fritschy and Grzanna, 1990). BDA (10% in saline) was injected iontophoretically, via a glass micropipette (25 μ m tip diameter), with 3 μ A positive current pulses (7 s on / 7 s off) for a period of 10 min.

Following tracer injections, the scalp was sutured and the animal was allowed to recover for 3 or 7 days after D-FITC and BDA injection, respectively.

Tissue preparation

At the end of the survival period, animals were anesthetized with a sodium pentobarbital overdose and then perfused transcardially with 100 ml of fresh Ringer's calcium-free buffer (NaCl, 145.45 mM; KCl, 3.35 mM; NaHCO₃, 2.38 mM), pH 6.9, at 37°C, followed by 1,000 ml of freshly depolymerized 4% paraformaldehyde in phosphate buffer 0.1 M, pH 7.4 (PB) at room temperature. For light microscopy (LM), the brains were removed and cryoprotected for 48 h at 4°C in 30% sucrose in PB. Serial coronal sections (40 μ m in thickness) were cut on a freezing stage sliding microtome (HM430; Microm, Heidelberg, Germany). The serial sections were collected in PB and were divided into a series of 10 bins. For confocal microscopy (CM), coronal brainstem sections of 40 μ m thickness were obtained with a Vibratome (#VT100S, Leica, Vienna, Austria).

Visualization of BDA tracer and immunohistochemistry for LM—BDA was revealed as previously described by López et al. (1999). In brief, free-floating sections were incubated with avidin-biotin-peroxidase complex (ABC, Standard-kit #PK 4000; Vector Laboratories, Burlingame, CA, USA), as outlined by Hsu et al. (1981), and revealed with 3,3'-diaminobenzidine tetrahydrochloride (DAB) and nickel ammonium sulfate (Hancock, 1982).

Standard immunostaining procedures were followed for the respective sections, which entailed incubating the sections for 72 h at 4°C in a primary antibody solution diluted in Tris-buffered saline containing 0.2% Triton X-100 (#T9284; Sigma, St. Louis, MO, USA). The tissue was then washed and incubated in its corresponding biotinylated secondary antibody for 2 h at room temperature. Complete information of primary and secondary antibodies is described in Table 1.

Following removal of the secondary antibodies, sections were incubated in ABC complex (Elite kit; #PK-6100, Vector Labs.) for 40 min at room temperature and revealed using DAB as the chromogen.

All sections were mounted on slides, dehydrated and coverslipped with Entellan® Neu (Merck). In addition, several series were counterstained with cresyl violet (0.1% #5235, Merck, Darmstadt, Germany) for cytoarchitectonic reference.

Visualization of BDA tracer and immunohistochemistry for CM—Alternating serial sections were used to immunolocalize VGLUT1 and VGAT. Free-floating sections were pretreated with phosphate buffer saline (PBS) 0.3% Triton X-100 and blocked for 1 h at room temperature with 6% normal goat serum (#S-1000, Vector Labs.) in PBS. After overnight incubation at 4 °C with primary antibodies, the sections were washed in PBS and incubated for 1 h at room temperature in the secondary antibody (Table 1).

In order to confirm the co-localization of tyrosine hydroxylase immunoreactive (TH-ir) and BDA-labeled fibers from the LC onto the CRNs, a triple labeling protocol was performed on several series of the BDA tracing experiments described above. The BDA was revealed by incubation in Cy2-conjugated streptavidin (1:650; #016-220-084; Jackson ImmunoResearch) together with a cocktail of antibodies against TH and calbindin D-28k to allow visualization of CRN somata and dendrites (López et al., 1993), as well as the presence of TH-ir fibers (Table 1). After a cocktail of secondary antibodies (Table 1), the sections were rinsed, mounted on slides and coverslipped with ProLong® Antifade Kit (#P7481; Molecular Probes). Negative controls were not treated with primary antibodies, and this resulted in no immunolabeling.

Isolation of RNA and RT-PCR

To study the presence of different receptor mRNAs, the cochlear nerve root of 14 rats was collected and homogenized. The prefrontal cortex (PFC) and hippocampus were also collected to serve as a control.

Total RNA was purified using the TRIZOL® reagent (Gibco BRL, Gaithersburg, MD, USA) following the manufacturer's protocol. RNA quality was assessed on an RNA 6000 NanoLabChip (#5065-4476; Agilent Technologies, Palo Alto, CA, USA), using an Agilent 2100 Bioanalyzer to assess the integrity of the 18S and 28S rRNA bands. Total RNA (5 µg), primed with oligo-dT, was reverse-transcribed into cDNA at 37 °C for 2 h using the first-strand cDNA synthesis kit (# 5800; Promega Corporation, Madison, WI, USA) in a 20 µl volume and stored at -20 °C until use, according to manufacturer's instructions. A 25 µl-PCR mixture contained 250 ng of cDNA template, 20 pmol of each primer (Additional file 1), 0.2 mM dNTPs, 1.5 mM MgCl₂ and 5 units of GoTaq Flexi DNA polymerase (# M8305; Promega Corp.).

PCRs were performed in an ABI7300 PCR system (#4351103; Applied Biosystems, Europe). The primers used, specific for each isoform, are listed in the Additional file 1. Amplification of rat β-actin (GenBank accession no. NM_031144) was used as an internal and loading control. PCR amplifications were as follows: 1 cycle at 95 °C for 5 min as an initial denaturation step, denaturation at 95 °C for 30 s, annealing at 51-59 °C for 30 s, and extension at 72 °C for 45 s (35 cycles), followed by further incubation for 10 min at 72 °C (1 cycle). PCR conditions were fixed in the linear phase of amplification to afford a semiquantitative analysis. PCR products were electrophoresed on 2.5% agarose gels in 1 X 40 mM Tris-acetate, 1 mM ethylenediamine tetraacetic acid, pH 8.0, and visualized by ethidium bromide staining.

To eliminate the possibility that amplified fragments might result from residual DNA rather than from RNA, the primers were designed in such a way that the RT-PCR products spanned two identified introns. Moreover, the amplification of β-actin was performed with equal amounts of RNA and revealed an identical expression pattern in all cerebral regions studied. Finally, in all RT-PCRs, an RNA-free (negative) control sample was used which did not produce any amplified bands.

Image Analysis

The terminology and abbreviations used to refer to the majority of brain nuclei were taken from the atlas of the rat brain of Paxinos and Watson (2005).

The sections processed for LM were studied using a microscope (#BX5; Olympus, Center Valley, PA, USA) equipped with a digital camera (Spot Rt[®]; Diagnostic Instruments, Sterling Heights, MI, USA). Low-magnification images were taken with a $\times 10$ objective lens and high-magnification images with $\times 60$ and $\times 100$ objective lenses (oil immersion).

Sections processed for CM were first examined using a microscope (#BX5; Olympus) equipped with fluorescence and a digital camera (Spot Rt[®]). Then, the sections were processed and analyzed in a Leica TCS SP2 confocal laser scanning microscope (Leica Microsystems, Mannheim, Germany) coupled to a Leica DM IRE2 inverted microscope and equipped with argon and helium neon lasers with excitation wavelengths of 458, 476, 488, 543, 568 and 633 nm. The fluorochromes FITC or Cy2, Cy3, and Cy5 were detected sequentially, stack-by-stack, with the acousto-optical tunable filter system and triple dichroic mirror TD488/543/633, using laser lines 488-495, 546 and 633 nm, respectively. The background was controlled and the photomultiplier voltage (800 V) was selected for maximum sensitivity in the linear range. The objectives used were Planapochromat $\times 20$, and oil immersion $\times 40$ and $\times 63$ /numerical aperture 1.30, giving a resolution of ~ 150 nm in the xy-plane and ~ 300 nm along the z-axis (pinhole 1 Airy unit), as well as several electronic zoom factors up to $\times 1.58$. To obtain a clear distribution of the labeled terminals on the cochlear root neurons, a series of 25 to 50 confocal images was obtained to generate a maximum-intensity z-projection of stacks, an orthogonal view (= xy, xz, yz planes for z-stacks series), and color coded image stacks based on volume. A sequence of 80 serial pictures from different viewpoints was created to produce three-dimensional (3D) animations. The movie documents generated from the image stacks were stored at 5 frames per second as AVI files. All z-projections and 3D animations were rendered using Leica confocal software (Leica Microsystems). All morphometry parameters were obtained using Scion Image software (Beta 4.02, NIH Image).

In addition, an intensity profile of the confocal image was defined to analyze the intensity values of the two channels using the MicroSuite 5 software (Olympus). The intensity profile ($14\ \mu\text{m}$) was drawn perpendicular to the cochlear root neuron dendrite bearing the labeled endings.

All photographs shown in the figures were processed with minor modifications in brightness and contrast using Adobe Photoshop[®] (version 9.0; Adobe Systems Incorporated, San Jose, CA, USA), and assembled using Canvas 7.0 software.

Results

Vesicular amino acid transporters on the CRNs

To examine the distribution of VGLUT1 and VGAT immunoreactivity in the cochlear root nucleus, we labeled CRNs by injecting D-FITC in the tz. We obtained large injection sites ($500\ \mu\text{m}$ in diameter) in the superficial portion of the tz, with little spreading into the reticular formation. Thick CRN axons ($\sim 3\ \mu\text{m}$ in diameter) coursing through the tz from the injection site were observed (Figs. 1A and 2E). Following the injections, the number of CRNs labeled retrogradely was 25-30, corresponding to 75 % of the entire population, and they were mostly located in the contralateral cochlear root nucleus. The retrograde labeling revealed a detailed morphology of the CRNs, including cell bodies and dendrites (Figs. 1C and 2H).

VGLUT1 immunoreactivity in the CRNs—We observed an intense, widespread immunoreactivity for VGLUT1 in the cochlear root nucleus (Fig. 1B, 1D and 1E). VGLUT1

immunoreactivity was in form of large and irregular puncta ($\sim 3 \mu\text{m}$ in diameter) majorly distributed on the CRN cell body surface, as well as covering dendrites (Fig. 1E, 1G, see also the on-line movie 1). Analysis of the intensity values confirmed that the VGLUT1-immunoreactive (VGLUT1-ir) endings were large, in close association with CRN dendrites (Fig. 1F), and that they mostly covered the entire dendrite on the z axis (Fig. G, on-line movie 2-3). We also quantified the number of VGLUT1-ir boutons along the length of twelve CRN dendrites and found that on average they received 25 endings per $100 \mu\text{m}$ dendrite length.

VGAT immunoreactivity in the CRNs—We found strong puncta immunoreactivity for VGAT in the cochlear root nucleus (Fig. 2). The distribution of VGAT immunoreactive (VGAT-ir) puncta in the cochlear root was different to that of VGLUT1-ir. The VGAT-ir puncta were smaller ($1.5 \mu\text{m}$ in diameter) and were distributed on CRN cell bodies as well as proximal and distal dendrites (Fig. 2B-2D and 2H). Interestingly, we observed a pool of CRNs in which VGAT-ir puncta were mainly distributed around the cell body (Fig. 2B-C). In contrast, on other CRNs, immunostaining was preferentially distributed around primary dendrites (Fig. 2D and 2G). The orthogonal view of serial z-stack images indicated that VGAT-ir endings were in close proximity to CRN cell bodies and dendrites (Fig. 2I). In addition, analysis of the intensity values for D-FITC labeled dendrites and the nearby VGAT-ir puncta corroborate that these endings terminate on dendrites (Fig. 2J).

Neurotransmitter receptor types in the cochlear root nucleus

To determine the types and subunits of excitatory and inhibitory neurotransmitter receptors in the cochlear root, we performed an analysis of neurotransmitter receptor gene expression in the cochlear root, together with an immunohistochemical study.

Gene expression of receptor types—We observed that the cochlear nerve root expressed several iGluRs, specifically: all members of the amino-3-hydroxy-5-methyl-isoxazole-4-propionate (AMPA) (GluR1-4) subtypes, at least six of the N-methyl-D-aspartate (NMDA) subtypes (NR1, NR2B-D and NR3A-B) and all the members of the kainate receptors (GluR5-7 and GluR δ 1-2) (Fig. 3). Moreover, we detected the expression of noradrenergic receptors (α 2A-C and β 1-3), muscarinic acetylcholine receptors (M1-5), and the glycine receptor α 1 (Fig. 3). Most of these genes have a marked expression in cells of the PFC and hippocampus, and hence could be used as positive control.

Interestingly, GABA_B receptors (BR1 and BR2) and the majority of the GABA_A subunit receptors (GABR α 1-6, β 1-3, γ 1-3 and θ) were expressed in the cochlear nerve root (Fig. 3). Other receptors subunits GABA_A (δ , π and ϵ) and GABA_C (ρ 1-3) were not found in the cochlear nerve root (data not shown). We also failed to detect the expression of glutamatergic-NMDA subtype NR2A in the cochlear nerve root or in the prefrontal cortex (Fig. 3).

The highest expression levels corresponded to the iGluRs: GluR1-2, NR2B-C, NR3A, GluR6 and GluR δ 2. The expression levels of muscarinic acetylcholine receptors (M1, M2, M4 and M5) and noradrenergic receptors (α 2A and β 3) were relatively high. PCR products corresponding to GluR3-4, NR1, NR2D, NR3B, GluR5, GluR7 and GluR δ 1 were relatively faint. Finally, cells of the cochlear nerve root slightly expressed at least four isoforms of GABA_A receptors (β 3 and γ 1-3), and moderately expressed GABA_A receptors (α 1-6, β 1-2 and θ) and GABA_B receptors (various BR1 isoforms and BR2) (Fig. 3).

Receptor Immunohistochemistry—CRNs receive direct synaptic contacts from glutamatergic synapses of the auditory nerve (Harrison et al., 1962; Brown et al., 1988; Merchán et al., 1988; Osen et al., 1991; Berglund et al., 1996). Our RT-PCR data detected AMPA and NMDA receptor subunits in the auditory nerve containing CRNs. To determine

the existence of glutamate receptors in CRNs, we determined the expression of the GluR1-4 subunits of the AMPA and the NR1 subunit of the NMDA glutamate receptors by immunohistochemistry. Our results showed a clear and distinct immunostaining of GluR3 and NR1 glutamate receptor types on the cell bodies and dendrites of CRNs (Fig. 4B, 4D, 4F). Cell body labeling for GluR1, GluR2 and GluR4 was considerably light or absent (Fig. 4A, 4C, 4E). The staining for GluR4 was observed in glial cells around unlabeled CRNs (Fig. 4E). Additionally, GluR4 and GFAP colocalized immunolabeling surrounding CRN cell bodies (Additional file 2), as previously reported (Conti et al., 1994).

As positive controls of the immunostaining, we also checked the labeling of these subunits in the anteroventral cochlear nucleus (AVCN) and cerebellum (data not shown). As previously shown in the adult AVCN, the staining for GluR1 and GluR2 was low while GluR4 and NR1 labeled different cell types (Petrálie et al., 2000). In the cerebellum, GluR1 and GluR4 only labeled Bergmann glial cells (Douyard et al., 2007), and GluR2 and NR1 staining was preferentially observed on Purkinje cells and in granule cells (data not shown; Petrálie et al., 1997; Thompson et al., 2000).

Glycine is considered the main inhibitory neurotransmitter in the cochlear nucleus, and the presence of glycinergic inputs on CRNs has been previously reported (Osen et al., 1991). Our RT-PCR data detected a positive signal for the $\alpha 1$ subunit of glycine receptors (GlyR $\alpha 1$) in the auditory nerve. Therefore, we used a specific antibody for this subunit to determine whether CRNs expressed glycine receptors. Immunostaining revealed that CRNs exhibit GlyR $\alpha 1$, and was predominately observed in the cell bodies (Fig. 4G).

Cholinergic innervation has been shown on CRN cell bodies and dendrites (Gómez-Nieto et al., 2008), and by RT-PCR we detected M2 and M4 in the auditory nerve as described above. On that account, we performed immunohistochemistry for M2 and M4 muscarinic receptor types. CRNs expressed both receptor types but differed in their pattern of immunostaining. We found weak M2 immunolabeling on CRN cell bodies and dendrites (Fig. 4H), as well as thin labeled axonal fibers and boutons surrounding dendritic and somatic profiles. On the contrary, M4 immunostaining was visible as multiple puncta on CRN cell bodies and dendrites (Fig. 4I). In the VCN magnocellular region a similar pre and postsynaptic pattern was previously reported (Yao et al., 1996).

Noradrenergic inputs to CRNs

TH immunohistochemistry of coronal sections revealed the strongest reactivity in the LC (Fig. 5A) and the presence of scant, thin TH-ir fibers bearing many varicosities in the cochlear nerve root. The TH-ir fibers were distributed along the lateral half of the cochlear root in the same region occupied by the CRNs (Fig. 5D, framed area); several of these terminal fibers were seen around the CRNs (Fig. 5E and 5G).

We injected BDA into the LC to confirm the input from this nucleus to the cochlear root (Fig. 5B and 5C). Anterogradely labeled axons coursed via two principal pathways: one crossed the parabrachial region toward the ventral cochlear nucleus and the CRNs on the same side of the injection, and the other coursed toward the contralateral PnC. The labeled fibers in the PnC gave rise to axon collaterals that crossed the midline, projecting to the contralateral ventral cochlear nucleus and cochlear nerve root.

The morphology of the BDA-labeled fibers (Fig. 5F and 5H) in the cochlear nerve root was exceptionally similar to that of the TH-ir fibers (Fig. 5E and 5G). These fibers were found in apposition to CRN somata and co-localized TH-ir and BDA, confirming the noradrenergic nature of the coeruleo-CRN projection (Fig. 5I). On the contralateral side of the BDA injections, occasional labeled fibers were seen in the cochlear nerve root (data not shown).

Discussion

The present study reports a novel noradrenergic projection from the LC to the CRNs and provides a well-defined differential description of VGLUT1 and VGAT immunoreactivity on the cell bodies and dendrites of rat CRNs. Using RT-PCR, we detected the expression of transcripts of several neurotransmitter receptors, and defined a molecular receptor profile of the cochlear nerve root consisting of: GluR1-4, NR1, NR2B-D, NR3A-B, GluR5-7, GluR δ 1-2, noradrenergic receptors (α 2A-C and β 1-3), muscarinic acetylcholine receptors (M1-5), GlyR α 1 subunit, GABA_A (α 1-6, β 1-3, γ 1-3, and θ), and GABA_B receptors (BR1-2). We also describe the immunoreactivity shown by the CRNs of the AMPA-GluR3, NMDA-NR1, GlyR α 1 subunits, and the muscarinic M2 and M4 receptor subunits. Taken together, our results provide valuable information about the convergence of various neurochemical inputs onto CRNs.

We observed negative immunostaining for several receptor subunits; nevertheless, the expression of their transcripts was detected with RT-PCR. The failure to identify these subunits by immunohistochemistry may have been due to the high sensitivity of the epitopes to the fixation technique (Fritschy et al. 1998), the inaccessibility of the epitopes to the antibodies, or a limitation in the sensitivity of the detection technique. However, a complete absence of the subunits on the CRNs cannot be ruled out.

Glutamatergic innervation in the CRNs

Cochlear root neurons receive many inputs from cochlear nerve fibers, but to date only CRN cell body synapses have been described (Harrison et al., 1962; Brown et al., 1988; Merchán et al., 1988; Osen et al., 1991; Berglund et al., 1996). Many studies have reported that cochlear nerve fibers use glutamate as their main neurotransmitter (Hackney et al., 1996; Trussell, 1999; Rubio and Juiz, 2004; Rubio, 2006; Zhou et al., 2007). Recently, it has been shown that VGLUT1 can be used as a reliable marker for glutamatergic terminals (Furness and Lawton, 2003; Zhou et al., 2007). In this study, we observed that VGLUT1 endings covered the cell bodies of CRNs, suggesting that they may receive inputs from cochlear nerve fibers. Interestingly, we also observed that VGLUT1 immunoreactive puncta terminated on retrogradely labeled CRN dendrites. By ultrastructural analysis (Merchán et al., 1988), it has been reported that auditory nerve fibers synapse onto the cell bodies of CRNs. Accordingly, our results indicate that auditory nerve fibers on cell bodies, and possibly on dendrites, use VGLUT1 to uptake glutamate into the synaptic vesicles.

As in the cochlear nuclear complex (Petralia et al., 2000; Rubio and Wenthold, 1997), glutamatergic neurotransmission within the CRNs may be mediated by AMPA and NMDA glutamate receptor types. Our results showed that CRNs were immunostained for AMPA-GluR3 receptor, but not for other AMPA receptor subunits, such as GluR1, GluR2 and GluR4. The lack of GluR2 in the CRNs indicates that AMPA receptor complexes on the cell body of CRNs might be Ca²⁺ permeable (Verdoorn et al., 1991; Rubio and Soto, 2001). This permeability to calcium would indicate the existence of an additional signal transduction pathway, in parallel with membrane depolarization, through which AMPA receptors can influence or control neural activity (Trussell, 1999). Given the preponderance of GluR3 in fast auditory synapses (Trussell, 1999), an AMPA receptor complex formed by GluR3 without GluR1, GluR2 or GluR4 might explain the fast and short-latency auditory responses of CRNs (Sinex et al., 2001). We also observed that CRNs were strongly immunostained for NMDA-NR1 receptor subunit. Many studies have shown the role of this glutamate receptor type in synaptic plasticity (Trussell, 1999). Recently, it has been reported that prepulse inhibition and habituation of the startle reflex is disrupted in mouse mutants with reduced numbers of the NMDA-NR1 receptor (Bickel et al., 2007). Taken together, the results of our study show that the cochlear root nucleus expresses a subset of glutamate receptor types and subunits that might

be necessary to provide fast auditory transmission for acoustic startle reflex elicitation and to allow the modulation or inhibition of CRN activity.

GABA/Glycinergic inhibition within the cochlear root nucleus

The VGAT localizes in the nerve endings of GABAergic and/or glycinergic innervation (McIntire et al., 1997; Chaudhry et al., 1998). Our results for VGAT immunolabeling revealed that VGAT-ir endings covered CRN cell bodies and dendrites, confirming the existence of GABAergic and glycinergic innervation (Osen et al., 1991). Ultrastructural analyses (Merchán et al., 1988; Gómez-Nieto et al., 2008) have also suggested the presence of inhibitory inputs on CRNs.

Our results revealed a strong immunoreactivity for the GlyR α 1 subunit on the cell bodies of CRNs. These data, together with the presence of glycinergic inputs on CRNs (Osen et al., 1991), advocate a role of glycine in CRN function. In addition, glycine may contribute to the inhibition of these neurons through the modulation of NMDA receptors (Furukawa et al., 2005). Moreover, it has been demonstrated that the NMDA receptor complexes associated with glycine receptors play an important role in the regulation of PPI (Furuya and Ogura, 1997).

It has been reported that GABA transmission is involved in PPI, in particular by activation of GABA_B receptors (Fendt et al., 2001; Takahashi et al., 2007). We found a variety of subunits of GABA_B and GABA_A receptors in the cochlear root, which could contribute to the inhibition of the ASR. Further studies need to be performed to determine whether CRNs express all or only a subset of the GABA receptor subtypes and subunits detected by RT-PCR. Another question that remains open is which projections to CRNs use GABA or glycine as their neurotransmitter. Among the potential sources of GABA/glycinergic inputs to CRNs are the superior olivary complex and/or other non-auditory structures yet to be identified.

Cholinergic modulation from the ventral nucleus of the trapezoid body

The presence of muscarinic receptors may imply a release of acetylcholine on CRNs. Many authors have reported cholinergic inputs of unknown origin to CRNs (Vetter et al., 1993; Yao and Godfrey, 1999), and recently cholinergic innervation from the VNTB has been shown on CRN cell bodies and dendrites (Gómez-Nieto et al., 2008). The neurotransmitter acetylcholine is present throughout the central nervous system, and its excitatory or inhibitory role depends on the postsynaptic receptors it acts upon. Of the five known subtypes of muscarinic receptors (M1-M5), only M2 and M4 are described as having an inhibitory effect on neurons (Brown et al., 1997; Caulfield and Birdsall, 1998), as occurs in reticulospinal neurons (Bosch and Schmid, 2006).

Our data showing that CRNs exhibit the M2 and M4 types, together with the existence of cholinergic inputs from the VNTB (Gómez-Nieto et al., 2008), suggest that acetylcholine may contribute to the inhibition of CRN response and, consequently, modulate the ASR by sound. In fact, it has been reported that acetylcholine plays a role in mediating PPI through M2 and M4 acetylcholine muscarinic receptors (Fendt et al., 2001; Bosch and Schmid, 2006). Whether this is also true in the auditory nerve nucleus remains to be further studied. The possibility that the cochlea-cochlear nucleus-VNTB-CRN pathway might relay fast auditory information that modulates the acoustic startle reflex in the auditory nerve may account for the peculiarities of PPI which are not explained with the current models of the PPI (Fendt et al., 2001; Yeomans et al., 2006). Although behavioral and electrophysiological studies are necessary to confirm the role of CRNs in the inhibition of the ASR, the present study provides further evidence that supports the role of the cochlear root nucleus in such inhibition.

Noradrenergic neurotransmission from the locus coeruleus

The expression of ADR α 2A and β 3 adrenergic subunit receptors in the cochlear root prompted us to examine the putative sources of noradrenergic innervation on CRNs. Previous studies have described the presence of noradrenergic fibers (Klepper and Herbert, 1991) and adrenergic receptors in the cochlear nuclei, but not in the extra-nuclear portion of the cochlear nerve (Wanaka et al., 1989; Domyancic and Morilak, 1997). The noradrenergic modulation of the ASR was early established by systemic administration of antagonists (Saitoh et al., 1986) and agonists of adrenergic receptors (Davis et al., 1977; Bakshi and Geyer, 1997). This modulation is mediated by central (Astrachan et al., 1983) but not peripheral receptors (Alsene et al., 2006).

The most important source of noradrenergic innervation to cochlear nuclei is the LC (Klepper and Herbert, 1991; Ebert, 1996), which also sends noradrenergic afferents to the PnC and VNTB (Fritschy and Grzanna, 1990; Mulders and Robertson, 2001). Some reports have suggested that the LC would modulate the ASR (Adams and Geyer, 1981; Von Coelln et al., 2004). In addition, the LC shows Fos expression after airpuff (tactile and acoustic) startle stimulation (Palmer and Printz, 1998). Accordingly, the LC seems to be connected to the two fundamental elements of the ASR: the PnC and the CRNs. The latter pathway is described in our study for the first time. We confirm the noradrenergic nature of the LC-CRNs pathway with the co-localization analysis of BDA and TH immunohistochemistry, which labels noradrenergic neurons in the LC (Klepper and Herbert, 1991; Aston-Jones, 2004). This pathway may contribute to noradrenergic modulation of the ASR, although other sources of noradrenergic afferents to CRNs may exist, such as observed in the noradrenergic innervation of cochlear nuclei (Klepper and Herbert, 1991). Furthermore, an indirect LC-CRNs connection through the cholinergic VNTB-CRNs pathway (Gómez-Nieto et al., 2008) might also participate in ASR modulation with a different neurochemical pattern.

The functional aspects of noradrenergic ASR modulation are not well understood because its effects depend on the receptor subtypes it acts upon (Davis et al., 1989; Bylund, 1992; Stevens et al., 1994; Carasso et al., 1998; Sallinen et al., 1998; Kable et al., 2000). In addition, evidence exists that noradrenaline can stimulate its receptors by paracrine transmission (Vizi et al., 2004).

Electrical stimulation of the LC results in an increase in spontaneous and tone-evoked discharge activity in the cochlear nucleus, comparable with iontophoretic administration of noradrenaline (Ebert, 1996). This effect may serve to increase the sensitivity of acoustic processing or to lower the threshold for short-latency acoustic reflexes such as the ASR.

Noradrenaline has been suggested to be involved in cochlear nucleus plasticity (Marien et al., 2004), which depends not only on the environment, but also on previous experiences. Studies have demonstrated that cochlear nucleus activity is modified by conditioning (Harvey et al., 1988; Woody et al., 1992) and behavioral state (May et al., 1998), and thus may be modulated by the noradrenergic system. Also, noradrenergic neurons are able to modulate cochlear neural output (Mulders and Robertson, 2005). The role of noradrenaline in the ASR by acting on the CRNs needs to be further explored.

In conclusion, we report for the first time the expression of cardinal neurotransmitter receptor types in the cochlear nerve root. We have expanded current knowledge of the glutamatergic, GABAergic, glycinergic, and cholinergic afferents to the CRNs, and have described a noradrenergic input to these neurons from the locus coeruleus. These results provide valuable elements to understanding the elicitation and synaptic modulation of the acoustic startle reflex.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Adams LM, Geyer MA. Effects of 6-hydroxydopamine lesions of locus coeruleus on startle in rats. *Psychopharmacology (Berl)* 1981;73:394–398. [PubMed: 6789365]
- Alsene KM, Carasso BS, Connors EE, Bakshi VP. Disruption of prepulse inhibition after stimulation of central but not peripheral alpha-1 adrenergic receptors. *Neuropsychopharmacology* 2006;31:2150–2161. [PubMed: 16407904]
- Andreski P, Chilcoat H, Breslau N. Post-traumatic stress disorder and somatization symptoms: A prospective study. *Psychiatry Research* 1998;79:131–138. [PubMed: 9705051]
- Anisman H, Kelly O, Hayley S, Borowski T, Merali Z, McIntyre DC. Acoustic startle and fear-potentiated startle in rats selectively bred for fast and slow kindling rates: Relation to monoamine activity. *European Journal of Neuroscience* 2000;12:4405–4416. [PubMed: 11122351]
- Aston-Jones, G. Locus Coeruleus, A5 and A7 noradrenergic cell groups. In: Paxinos, G., editor. *The rat nervous system*. San Diego: Elsevier; 2004. p. 259-294.
- Astrachan DI, Gallager DW, Davis M. Behavior and binding: desensitization to alpha 1-adrenergic stimulation of acoustic startle is associated with a decrease in alpha 1-adrenoceptor binding sites. *Brain Res* 1983;276:183–187. [PubMed: 6313129]
- Bakshi VP, Geyer MA. Phencyclidine-induced deficits in prepulse inhibition of startle are blocked by prazosin, an alpha-1 noradrenergic antagonist. *The Journal of pharmacology and experimental therapeutics* 1997;283:666–674. [PubMed: 9353384]
- Bakshi VP, Swerdlow NR, Geyer MA. Clozapine antagonizes phencyclidine-induced deficits in sensorimotor gating of the startle response. *Journal of Pharmacology and Experimental Therapeutics* 1994;271:787–794. [PubMed: 7965797]
- Bell RL, Rodd ZA, Hsu CC, Lumeng L, Murphy JM, McBride WJ. Amphetamine-modified acoustic startle responding and prepulse inhibition in adult and adolescent alcohol-preferring and -nonpreferring rats. *Pharmacol Biochem Behav* 2003;75:163–171. [PubMed: 12759124]
- Berglund AM, Benson TE, Brown MC. Synapses from labeled type II axons in the mouse cochlear nucleus. *Hear Res* 1996;94:31–46. [PubMed: 8789809]
- Bickel S, Lipp HP, Umbricht D. Early Auditory Sensory Processing Deficits in Mouse Mutants with Reduced NMDA Receptor Function. *Neuropsychopharmacology*. 200710.1038/sj.npp.1301536 Advance online publication 22 August 2007
- Bosch D, Schmid S. Activation of muscarinic cholinergic receptors inhibits giant neurones in the caudal pontine reticular nucleus. *European Journal of Neuroscience* 2006;24:1967–1975. [PubMed: 17040474]
- Braff DL, Geyer MA, Swerdlow NR. Human studies of prepulse inhibition of startle: normal subjects, patient groups, and pharmacological studies. *Psychopharmacology (Berl)* 2001;156:234–258. [PubMed: 11549226]
- Brown DA, Abogadie FC, Allen TGJ, Buckley NJ, Caulfield MP, Delmas P, Haley JE, Lamas JA, Selyanko AA. Muscarinic mechanisms in nerve cells. *Life sciences* 1997;60:1137–1144. [PubMed: 9121358]
- Brown MC, Berglund AM, Kiang NY, Ryugo DK. Central trajectories of type II spiral ganglion neurons. *J Comp Neurol* 1988;278:581–590. [PubMed: 3230171]

- Bylund DB. Subtypes of alpha 1- and alpha 2-adrenergic receptors. *Faseb J* 1992;6:832–839. [PubMed: 1346768]
- Carasso BS, Bakshi VP, Geyer MA. Disruption in prepulse inhibition after alpha-1 adrenoceptor stimulation in rats. *Neuropharmacology* 1998;37:401–404. [PubMed: 9681939]
- Caulfield MP, Birdsall NJ. International Union of Pharmacology. XVII. Classification of muscarinic acetylcholine receptors. *Pharmacol Rev* 1998;50:279–290. [PubMed: 9647869]
- Chaudhry FA, Reimer RJ, Bellocchio EE, Danbolt NC, Osen KK, Edwards RH, Storm-Mathisen J. The vesicular GABA transporter, VGAT, localizes to synaptic vesicles in sets of glycinergic as well as GABAergic neurons. *J Neurosci* 1998;18:9733–9750. [PubMed: 9822734]
- Conti F, Minelli A, Brecha NC. Cellular localization and laminar distribution of AMPA glutamate receptor subunits and mRNAs and proteins in the rat cerebral cortex. *J Comp Neurol* 1994;350:241–259. [PubMed: 7884041]
- Davis M. Neural systems involved in fear and anxiety measured with fear-potentiated startle. *American Psychologist* 2006;61:741–756. [PubMed: 17115806]
- Davis M, Cedarbaum JM, Aghajanian GK, Gendelman DS. Effects of clonidine on habituation and sensitization of acoustic startle in normal, decerebrate and locus coeruleus lesioned rats. *Psychopharmacology (Berl)* 1977;51:243–253. [PubMed: 403539]
- Davis M, Commissaris RL, Yang S, Wagner KR, Kehne JH, Cassella JV, Boulis NM. Spinal vs. supraspinal sites of action of the alpha 2-adrenergic agonists clonidine and ST-91 on the acoustic startle reflex. *Pharmacol Biochem Behav* 1989;33:233–240. [PubMed: 2571168]
- Domyancic AV, Morilak DA. Distribution of alpha1A adrenergic receptor mRNA in the rat brain visualized by in situ hybridization. *J Comp Neurol* 1997;386:358–378. [PubMed: 9303423]
- Douyard J, Shen L, Haganir RL, Rubio ME. Differential neuronal and glial expression of GluR1 AMPA receptor subunit, SAP97 and 4.1N during cerebellar development. *J Comp Neurol* 2007;502:141–156. [PubMed: 17335044]
- Ebert U. Noradrenaline enhances the activity of cochlear nucleus neurons in the rat. *Eur J Neurosci* 1996;8:1306–1314. [PubMed: 8752601]
- Fendt M, Koch M. Cholinergic modulation of the acoustic startle response in the caudal pontine reticular nucleus of the rat. *Eur J Pharmacol* 1999;370:101–107. [PubMed: 10323257]
- Fendt M, Li L, Yeomans JS. Brain stem circuits mediating prepulse inhibition of the startle reflex. *Psychopharmacology (Berl)* 2001;156:216–224. [PubMed: 11549224]
- Fritschy JM, Grzanna R. Distribution of locus coeruleus axons within the rat brainstem demonstrated by Phaseolus vulgaris leucoagglutinin anterograde tracing in combination with dopamine-beta-hydroxylase immunofluorescence. *J Comp Neurol* 1990;293:616–631. [PubMed: 2329197]
- Fritschy JM, Weinmann O, Wenzel A, Benke D. Synapse-specific localization of NMDA and GABA(A) receptor subunits revealed by antigen-retrieval immunohistochemistry. *J Comp Neurol* 1998;390:194–210. [PubMed: 9453664]
- Furness DN, Lawton DM. Comparative Distribution of Glutamate Transporters and Receptors in Relation to Afferent Innervation Density in the Mammalian Cochlea. *Journal of Neuroscience* 2003;23:11296–11304. [PubMed: 14672993]
- Furukawa H, Singh SK, Mancusso R, Gouaux E. Subunit arrangement and function in NMDA receptors. *Nature* 2005;438:185–192. [PubMed: 16281028]
- Furuya Y, Ogura H. Competitive NMDA and strychnine-insensitive glycine-site antagonists disrupt prepulse inhibition. *Pharmacol Biochem Behav* 1997;57:909–913. [PubMed: 9259023]
- Geyer MA, Krebs-Thomson K, Braff DL, Swerdlow NR. Pharmacological studies of prepulse inhibition models of sensorimotor gating deficits in schizophrenia: A decade in review. *Psychopharmacology* 2001;156:117–154. [PubMed: 11549216]
- Gómez-Nieto R, Rubio ME, López DE. Cholinergic input from the ventral nucleus of the trapezoid body to cochlear root neurons in rats. *J Comp Neurol* 2008;506:452–468. [PubMed: 18041785]
- Gonzalez-Lima F, Finkenstadt T, Ewert JP. Neural substrates for long-term habituation of the acoustic startle reflex in rats: a 2-deoxyglucose study. *Neurosci Lett* 1989;96:151–156. [PubMed: 2927718]
- Guyenet PG. The coeruleospinal noradrenergic neurons: anatomical and electrophysiological studies in the rat. *Brain Res* 1980;189:121–133. [PubMed: 7363081]

- Hackney CM, Osen KK, Ottersen OP, Storm-Mathisen J, Manjaly G. Immunocytochemical evidence that glutamate is a neurotransmitter in the cochlear nerve: a quantitative study in the guinea-pig anteroventral cochlear nucleus. *Eur J Neurosci* 1996;8:79–91. [PubMed: 8713452]
- Hancock MB. DAB-Nikel Substrate for the differential immunoperoxidase staining of nerve fibers and fiber terminals. *J Histochem Cytochem* 1982;30:578.
- Harrison JM, Warr WB, Irving RE. Second order neurons in the acoustic nerve. *Science* 1962;138:893–895. [PubMed: 13952991]
- Harvey JA, Winsky L, Schindler CW, McMaster SE, Welsh JP. Asymmetric uptake of 2-deoxy-D-[14C] glucose in the dorsal cochlear nucleus during Pavlovian conditioning in the rabbit. *Brain Res* 1988;449:213–224. [PubMed: 3395846]
- Hoffman HS, Fleshler M. Startle reaction: Modification by background acoustic stimulation. *Science* 1963;141:928–930. [PubMed: 14043340]
- Hoffman HS, Searle JL. Acoustic variables in the modification of startle reaction in the rat. *J Comp Physiol Psychol* 1965;60:53–58. [PubMed: 14334243]
- Hsu SM, Raine L, Fanger H. The use of antiavidin antibody and avidin-biotin-peroxidase complex in immunoperoxidase technics. *Am J Clin Pathol* 1981;75:816–821. [PubMed: 6167159]
- Kable JW, Murrin LC, Bylund DB. In vivo gene modification elucidates subtype-specific functions of alpha(2)-adrenergic receptors. *The Journal of pharmacology and experimental therapeutics* 2000;293:1–7. [PubMed: 10734146]
- Kaviani H, Gray JA, Checkley SA, Raven PW, Wilson GD, Kumari V. Affective modulation of the startle response in depression: Influence of the severity of depression, anhedonia, and anxiety. *Journal of Affective Disorders* 2004;83:21–31. [PubMed: 15546642]
- Klepper A, Herbert H. Distribution and origin of noradrenergic and serotonergic fibers in the cochlear nucleus and inferior colliculus of the rat. *Brain Res* 1991;557:190–201. [PubMed: 1747753]
- Koch M. The neurobiology of startle. *Prog Neurobiol* 1999;59:107–128. [PubMed: 10463792]
- Koch M, Lingenhöhl K, Pilz PK. Loss of the acoustic startle response following neurotoxic lesions of the caudal pontine reticular formation: possible role of giant neurons. *Neuroscience* 1992;49:617–625. [PubMed: 1386915]
- Koch M, Schnitzler HU. The acoustic startle response in rats--circuits mediating evocation, inhibition and potentiation. *Behav Brain Res* 1997;89:35–49. [PubMed: 9475613]
- Lee Y, López DE, Meloni EG, Davis M. A primary acoustic startle pathway: obligatory role of cochlear root neurons and the nucleus reticularis pontis caudalis. *J Neurosci* 1996;16:3775–3789. [PubMed: 8642420]
- Lingenhöhl K, Friauf E. Giant neurons in the rat reticular formation: a sensorimotor interface in the elementary acoustic startle circuit? *J Neurosci* 1994;14:1176–1194. [PubMed: 8120618]
- López, DE.; Merchán, MA.; Bajo, VM.; Saldana, E. The cochlear root neurons in the rat, mouse and gerbil. In: Merchán, MA., editor. *The mammalian cochlear nuclei: organization and function*. New York: Plenum Press; 1993. p. 291-301.
- López DE, Saldana E, Nodal FR, Merchán MA, Warr WB. Projections of cochlear root neurons, sentinels of the rat auditory pathway. *J Comp Neurol* 1999;415:160–174. [PubMed: 10545157]
- Marien MR, Colpaert FC, Rosenquist AC. Noradrenergic mechanisms in neurodegenerative diseases: a theory. *Brain Res Brain Res Rev* 2004;45:38–78. [PubMed: 15063099]
- May BJ, Prell GS, Sachs MB. Vowel representations in the ventral cochlear nucleus of the cat: effects of level, background noise, and behavioral state. *J Neurophysiol* 1998;79:1755–1767. [PubMed: 9535945]
- McIntire SL, Reimer RJ, Schuske K, Edwards RH, Jorgensen EM. Identification and characterization of the vesicular GABA transporter. *Nature* 1997;389:870–876. [PubMed: 9349821]
- Merchán MA, Collia F, López DE, Saldana E. Morphology of cochlear root neurons in the rat. *J Neurocytol* 1988;17:711–725. [PubMed: 2463341]
- Mulders WH, Robertson D. Origin of the noradrenergic innervation of the superior olivary complex in the rat. *J Chem Neuroanat* 2001;21:313–322. [PubMed: 11429272]
- Mulders WHAM, Robertson D. Noradrenergic modulation of brainstem nuclei alters cochlear neural output. *Hearing Research* 2005;204:147–155. [PubMed: 15925200]

- Nodal FR, López DE. Direct input from cochlear root neurons to pontine reticulospinal neurons in albino rat. *J Comp Neurol* 2003;460:80–93. [PubMed: 12687698]
- Osen KK, López DE, Slyngstad TA, Ottersen OP, Storm-Mathisen J. GABA-like and glycine-like immunoreactivities of the cochlear root nucleus in rat. *J Neurocytol* 1991;20:17–25. [PubMed: 1709203]
- Palmer AA, Printz MP. Attenuation of Fos expression to airpuff startle stimuli following tympanic membrane rupture. *Brain Res* 1998;787:91–98. [PubMed: 9518564]
- Paxinos, G.; Watson, C. *The Rat Brain in Stereotaxic Coordinates: the new coronal set - 161 diagrams*. San Diego: Academic Press; 2005.
- Petralia RS, Wang YX, Mayat E, Wenthold RJ. Glutamate receptor subunit 2-selective antibody shows a differential distribution of calcium-impermeable AMPA receptors among populations of neurons. *J Comp Neurol* 1997;385:456–476. [PubMed: 9300771]
- Petralia RS, Rubio ME, Wang YX, Wenthold RJ. Differential distribution of glutamate receptors in the cochlear nuclei. *Hearing Research* 2000;147:59–69. [PubMed: 10962173]
- Pilz PK, Schnitzler HU. Habituation and sensitization of the acoustic startle response in rats: amplitude, threshold, and latency measures. *Neurobiol Learn Mem* 1996;66:67–79. [PubMed: 8661252]
- Prehn A, Ohrt A, Sojka B, Ferstl R, Pause BM. Chemosensory anxiety signals augment the startle reflex in humans. *Neuroscience Letters* 2006;394:127–130. [PubMed: 16257486]
- Rubio ME. Redistribution of synaptic AMPA receptors at glutamatergic synapses in the dorsal cochlear nucleus as an early response to cochlear ablation in rats. *Hearing Research* 2006;216–217:154–167. [PubMed: 16644159]
- Rubio ME, Juiz JM. Differential distribution of synaptic endings containing glutamate, glycine, and GABA in the rat dorsal cochlear nucleus. *J Comp Neurol* 2004;477:253–272. [PubMed: 15305363]
- Rubio ME, Soto F. Distinct Localization of P2X receptors at excitatory postsynaptic specializations. *J Neurosci* 2001;21:641–653. [PubMed: 11160443]
- Rubio ME, Wenthold RJ. Glutamate receptors are selectively targeted to postsynaptic sites in neurons. *Neuron* 1997;18:939–950. [PubMed: 9208861]
- Saitoh K, Shaw S, Tilson HA. Noradrenergic influence on the prepulse inhibition of acoustic startle. *Toxicology letters* 1986;34:209–216. [PubMed: 3798479]
- Sallinen J, Haapalinna A, Viitamaa T, Kobilka BK, Scheinin M. Adrenergic alpha2C-receptors modulate the acoustic startle reflex, prepulse inhibition, and aggression in mice. *J Neurosci* 1998;18:3035–3042. [PubMed: 9526020]
- Sinex DG, López DE, Warr WB. Electrophysiological responses of cochlear root neurons. *Hear Res* 2001;158:28–38. [PubMed: 11506934]
- Stam R. PTSD and stress sensitisation: A tale of brain and body. Part 1: Human studies. *Neuroscience and Biobehavioral Reviews* 2007;31:530–557. [PubMed: 17270271]
- Stevens DR, McCarley RW, Greene RW. The mechanism of noradrenergic alpha 1 excitatory modulation of pontine reticular formation neurons. *J Neurosci* 1994;14:6481–6487. [PubMed: 7965052]
- Swerdlow NR, Blumenthal TD, Sutherland AN, Weber E, Talledo JA. Effects of prepulse intensity, duration, and bandwidth on perceived intensity of startling acoustic stimuli. *Biol Psychol* 2007;74:389–395. [PubMed: 17118517]
- Swerdlow NR, Braff DL, Geyer MA. Animal models of deficient sensorimotor gating: what we know, what we think we know, and what we hope to know soon. *Behav Pharmacol* 2000;11:185–204. [PubMed: 11103873]
- Swerdlow NR, Geyer MA. Clozapine and haloperidol in an animal model of sensorimotor gating deficits in schizophrenia. *Pharmacology Biochemistry and Behavior* 1993;44:741–744.
- Takahashi K, Nagai T, Kamei H, Maeda K, Matsuya T, Arai S, Mizoguchi H, Yoneda Y, Nabeshima T, Takuma K, Yamada K. Neural circuits containing pallidotegmental GABAergic neurons are involved in the prepulse inhibition of the startle reflex in mice. *Biological psychiatry* 2007;62:148–157. [PubMed: 17027927]
- Thompson AM. Pontine sources of norepinephrine in the cat cochlear nucleus. *Journal of Comparative Neurology* 2003;457:374–383. [PubMed: 12561077]

- Thompson CL, Drewery DL, Atkins HD, Stephenson FA, Chazot PL. Immunohistochemical localization of N-methyl-D-aspartate receptor NR1, NR2A, NR2B, and NR2C/D subunits in the adult mammalian cerebellum. *Neuroscience Letters* 2000;283:85–88. [PubMed: 10739881]
- Trussell LO. Synaptic mechanisms for coding timing in auditory neurons. *Annu Rev Physiol* 1999;61:477–496. [PubMed: 10099698]
- Verdoorn TA, Burnashev N, Monyer H, Seeburg PH, Sakmann B. Structural determinants of ion flow through recombinant glutamate receptor channels. *Science* 1991;252:1715–1718. [PubMed: 1710829]
- Vetter DE, Saldana E, Mugnaini E. Input from the inferior colliculus to medial olivocochlear neurons in the rat: a double label study with PHA-L and cholera toxin. *Hear Res* 1993;70:173–186. [PubMed: 8294262]
- Vizi ES, Kiss JP, Lendvai B. Nonsynaptic communication in the central nervous system. *Neurochemistry international* 2004;45:443–451. [PubMed: 15186910]
- Von Coelln R, Thomas B, Savitt JM, Lim KL, Sasaki M, Hess EJ, Dawson VL, Dawson TM. Loss of locus coeruleus neurons and reduced startle in parkin null mice. *Proc Natl Acad Sci U S A* 2004;101:10744–10749. [PubMed: 15249681]
- Wanaka A, Kiyama H, Murakami T, Matsumoto M, Kamada T, Malbon CC, Tohyama M. Immunocytochemical localization of beta-adrenergic receptors in the rat brain. *Brain Res* 1989;485:125–140. [PubMed: 2541863]
- Winslow JT, Noble PL, Davis M. Modulation of Fear-Potentiated Startle and Vocalizations in Juvenile Rhesus Monkeys by Morphine, Diazepam, and Buspirone. *Biological psychiatry* 2007;61:389–395. [PubMed: 16730332]
- Woody CD, Wang XF, Gruen E, Landeira-Fernandez J. Unit activity to click CS changes in dorsal cochlear nucleus after conditioning. *Neuroreport* 1992;3:385–388. [PubMed: 1633272]
- Yao W, Godfrey DA, Levey AI. Immunolocalization of muscarinic acetylcholine subtype 2 receptors in rat cochlear nucleus. *J Comp Neurol* 1996;373(1):27–40. [PubMed: 8876460]
- Yao W, Godfrey DA. Vesicular acetylcholine transporter in the rat cochlear nucleus: an immunohistochemical study. *J Histochem Cytochem* 1999;47:83–90. [PubMed: 9857215]
- Yeomans JS, Frankland PW. The acoustic startle reflex: neurons and connections. *Brain Res Brain Res Rev* 1996;21:301–314. [PubMed: 8806018]
- Yeomans JS, Lee J, Yeomans MH, Steidl S, Li L. Midbrain pathways for prepulse inhibition and startle activation in rat. *Neuroscience* 2006;142:921–929. [PubMed: 16996220]
- Zhao Z, Davis M. Fear-potentiated startle in rats is mediated by neurons in the deep layers of the superior colliculus/deep mesencephalic nucleus of the rostral midbrain through the glutamate non-NMDA receptors. *J Neurosci* 2004;24:10326–10334. [PubMed: 15548646]
- Zhou J, Nannapaneni N, Shore S. Vesicular glutamate transporters 1 and 2 are differentially associated with auditory nerve and spinal trigeminal inputs to the cochlear nucleus. *J Comp Neurol* 2007;500:777–787. [PubMed: 17154258]

Abbreviations

ABC	avidin-biotin-peroxidase complex
ADR	noradrenergic receptor
AMPA	amino-3-hydroxy-5-methyl-isoxazole-4-propionate
ASR	acoustic startle reflex
AVCN	anteroventral cochlear nucleus

BDA	biotinylated dextran amine
CRN	cochlear root neuron
CM	confocal microscopy
DAB	3,3' diaminobenzidine tetrahydrochloride
D-FITC	fluorescein isothiocyanate-conjugated dextran
GABA	gamma-aminobutyric acid
GABR	GABA receptor
GluR	glutamate receptor
GlyR	glycine receptor
iGluR	ionotropic glutamate receptor
LC	locus coeruleus
LM	light microscopy
NMDA	N-methyl-D-aspartate
NR	glutamatergic-NMDA receptor
PB	phosphate buffer 0.1 M, pH 7.4
PBS	phosphate buffer saline 0.1 M, pH 7.4
PFC	prefrontal cortex
PnC	caudal pontine reticular nucleus
PPI	prepulse inhibition
RT-PCR	reverse transcription-polymerase chain reaction

TH	tyrosine hydroxylase
TH-ir	tyrosine hydroxylase-immunoreactive
tz	trapezoid body
VGAT	vesicular gamma-aminobutyric acid transporter
VGAT-ir	vesicular gamma-aminobutyric acid transporter-immunoreactive
VGLUT1	vesicular glutamate transporter subtype 1
VGLUT1-ir	VGLUT-immunoreactive
VNTB	ventral nucleus of the trapezoid body

Figure 1.

VGLUT1 immunoreactivity in the cochlear root nucleus. **A:** CRNs retrogradely labeled with D-FITC. Notice large-diameter CRN axons (arrows) coursing through the tz from the injection site. **B:** Magnification of framed area in A. VGLUT1-ir boutons (red) on CRNs retrogradely labeled with D-FITC (green). Intense VGLUT1 immunoreactive puncta are observed on the CRNs. **C-D-E:** High magnification of the square in B showing VGLUT1-ir endings on cell bodies (arrows) and dendrites (arrowheads) of CRNs. **C** (D-FITC, green), **D** (VGLUT1, red), **E** (merged) see also the on-line movie 1. **F:** Graph of the intensity values for red and green channels along the line 1 drawn in E. The large VGLUT1-ir endings (red line) terminating on the CRN dendrites (green line). **G:** Coded color projection of two CRNs corresponding to the

asterisks in E. VGLUT1-ir endings entirely cover the surface of CRN cell bodies and primary dendrites. Volumes are color coded from blue (0 μm) to orange (+ 40 μm) as shown in the color bar. See also the on-line movie 2 and 3. VGLUT1-ir, VGLUT1-immunoreactive; CRNs, cochlear root neurons; D-FITC, fluorescein isothiocyanate-conjugated dextran; tz, trapezoid body; VGLUT1, vesicular glutamate transporter subtype 1.

Figure 2.

VGAT immunoreactivity in the CRNs. **A:** Low magnification epifluorescence image showing VGAT immunolabeling in the cochlear root nucleus. **B-C-D:** Detail images from dorsal to ventral regions of the cochlear root nucleus corresponding to the squares in A. Notice VGAT-ir endings (arrowheads) terminating on CRNs. **E:** CRNs retrogradely labeled with D-FITC. Thick-diameter CRN axons (arrows) course through the tz. **F-G-H:** High magnification ($\times 40$, square in E) showing VGAT-ir bouton-like terminations on cell bodies (arrows) and dendrites (arrowheads) of CRNs. **F** (D-FITC, green), **G** (VGAT, red), **H** (merged). **I:** Orthogonal view of the merged image showing VGAT-ir terminals in apposition to the CRN dendrite through the Z-projection. **J:** Graph of the intensity values for red and green channels along line 1 drawn in I. Small VGAT-ir endings (red line) terminating on the CRN dendrite (green line). VGAT-

ir, VGAT-immunoreactive; CRNs, cochlear root neurons; D-FITC, fluorescein isothiocyanate-conjugated dextran; tz, trapezoid body; VGAT, vesicular GABA transporter.

Figure 3.

Different expression levels of ionotropic glutamate receptors [AMPA (GluR1-4), NMDA (NR1, NR2A-D and NR3A-B) and kainate (GluR5-7 and GluRδ1-2)], noradrenergic receptors, muscarinic acetylcholine receptors, glycine receptor α1 and GABA receptors in cells of the cochlear nerve root (CNR), prefrontal cortex (PFC), hippocampus (H) and negative control (C-) in the exponential phase of different PCRs (35 cycles). Sequence names, primer sequences, sizes of the different fragments, and conditions of PCRs are detailed in Material and Methods and in the Additional file 1. In GABA_B-BR1, we detected the expression of the c isoform and at least one of the isoforms a, b or d. β-actin was used as a control for equal amounts in RT-PCR reaction.

Figure 4.
Photomicrographs of neurotransmitter receptor immunostaining in the cochlear root nucleus. **A to E:** AMPA (GluR1-4), **F:** NMDA (NR1) glutamate receptors, **G:** glycine receptor $\alpha 1$ subunit (GlyR $\alpha 1$), **H and I:** muscarinic receptor types (M2 and M4). Insets correspond to higher magnifications of CRNs.

Figure 5.

Noradrenergic input from the locus coeruleus (LC) to CRNs. **A:** TH immunoreactivity (arrow) in the LC. **B:** Typical injection site of BDA (arrow head) in the LC. **C:** Camera lucida drawing indicating the BDA injection sites in each case. **D:** Coronal section of cochlear root, Nissl-counterstained, to show the localization of CRNs in the framed area. **E** and **G:** TH-ir fibers around the unlabeled CRNs (arrow). **F** and **H:** BDA labeled fibers around the unlabeled CRNs (arrow head). **I:** Co-localization of BDA labeled (arrow head) and TH-ir fibers (arrow) in apposition to CRN soma. BDA, biotinylated dextran amine.

List of antibodies and dilutions used in the immunohistochemical experiments. Ale, Alexis Biochemicals, San Diego, CA, USA; Chem, Chemicon, Temecula, CA, USA; Dia, Diasorin, Stillwater, MN, USA; Jac, Jackson Immunoresearch, West Grove, PA, USA; Sw, Swant, Bellinzona, Switzerland; Sy, Synaptic Systems, Goettingen, Germany; Vec, Vector Laboratories, Burlingame, CA, USA; LM, Light microscopy; CM, Confocal microscopy.

Table 1

Antigen	Primary AB	Reference	Dilution	Secondary AB	Reference	Dilution	Method
AMPA-GluR 1	rabbit anti GLuR1	#AB1504-Chem	1/1000	biotinylated goat anti-rabbit	#BA-1000-Vec		
AMPA-GluR 2	rabbit anti-GLuR 2	#AB9209-Chem					
AMPA-GluR 2/3	rabbit anti-GLuR 2/3	#AB1506-Chem					
AMPA-GluR 4	rabbit anti-GLuR 4	#AB1508-Chem					
NMDA-NR1	rabbit anti-NR1	#AB5046P-Chem					
muscarinic M2	rat anti-M2	#MAB367-Chem	1/2000	Biotinylated rabbit anti-rat	#BA-2001-Vec	1/200	LM
muscarinic M4	mouse anti-M4	#MAB1576-Chem	1/1500	biotinylated horse anti-mouse	#BA-4000-Vec		
GlyR a1	mouse anti-GlyR a1	#804-037-C100-Ale					
TH	mouse anti-TH	#22941-Dia	1/100	biotinylated goat anti-mouse	#BA-2000-Vec	1/250	
VGLuT-1	rabbit anti VGLuT-1	#135302-Sy	1/1000	Cy5 anti-mouse IgG	#115-175-003-Jac		
VGAT	rabbit anti VGAT	#AB5062P-Chem				1/200	CM
CaBP D-28K	rabbit anti-CaBP D-28K	CB-38a-Sw	1/5000	Cy3 goat anti-rabbit	#111-165-003-Jac	1/250	