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Effects of cochlear ablation on amino acid levels in the rat cochlear nucleus and superior olive

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Abstract

Amino acids have important roles in the chemistry of the auditory system, including communication among neurons. There is much evidence for glutamate as a neurotransmitter from auditory nerve fibers to cochlear nucleus neurons. Previous studies in rodents have examined effects of removal of auditory nerve input by cochlear ablation on levels, uptake and release of glutamate in cochlear nucleus subdivisions, as well as on glutamate receptors. Effects have also been reported on uptake and release of γ -aminobutyrate (GABA) and glycine, two other amino acids strongly implicated in cochlear nucleus synaptic transmission. We mapped the effects of cochlear ablation on the levels of amino acids, including glutamate, GABA, glycine, aspartate, glutamine, taurine, serine, threonine, and arginine, in microscopic subregions of the rat cochlear nucleus. Submicrogram-size samples microdissected from freeze-dried brainstem sections were assayed for amino acid levels by high performance liquid chromatography. After cochlear ablation, glutamate and aspartate levels decreased by 2 days in regions receiving relatively dense innervation from the auditory nerve, whereas the levels of most other amino acids increased. The results are consistent with a close association of glutamate and aspartate with auditory nerve fibers and of other amino acids with other neurons and glia in the cochlear nucleus. A consistent decrease of GABA level in the lateral superior olive could be consistent with a role in some lateral olivocochlear neurons. The results are compared with those obtained with the same methods for the rat vestibular nerve root and nuclei after vestibular ganglionectomy.

Keywords

aspartate; auditory; GABA; glycine; glutamate; taurine

1. Introduction

Damage to peripheral sensory organs can lead to changes at multiple levels in the central nervous system, including changes in chemistry (Wall et al., 2002). In the auditory system,

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damage to the cochlea part of the inner ear can result in hearing loss and distorted hearing, such as tinnitus (Eggermont, 2012). Chemical changes associated with these deficits have been studied at many levels of the rodent auditory system (Fisher and Davies, 1976; Wenthold and Gulley, 1977; Wenthold, 1978, 1979; Ryan et al., 1992; Hackney et al., 1996; Abbott et al., 1999; Milbrandt et al., 2000; Potashner et al., 1985, 1997, 2000; Suneja et al., 1998a,b, 2000; Michler and Illing, 2003; Zhang et al., 2003a,b; Jin et al., 2005, 2006; Jin and Godfrey, 2006; Godfrey et al., 2008a,b, 2012, 2013; Wang et al., 2009, 2011; Dong et al., 2010; Hildebrandt et al., 2011; Kraus et al., 2011; Fredrich et al., 2013), but the most immediate effects occur at its earliest levels, where the auditory nerve carries information about sounds from the cochlea to the cochlear nucleus (Harrison and Warr, 1962; Harrison and Irving, 1965, 1966; Merchán et al., 1985). The superior olivary complex, which receives extensive input from the cochlear nucleus, is also connected to the cochlea via a centrifugal pathway, the olivocochlear bundle (Warr, 1992).

Previous studies in rodents have indicated important functions for the amino acids glutamate, glycine, and γ -aminobutyrate (GABA) as major neurotransmitters in the cochlear nucleus (Fisher and Davies, 1976; Wenthold and Gulley, 1977; Wenthold, 1978, 1979; Hackney et al., 1996; Potashner et al., 1985, 1997, 2000; Suneja et al., 1998a,b, 2000; Godfrey et al., 2008a,b, 2012; Wang et al., 2009; Dong et al., 2010). Glutamate is strongly supported as a neurotransmitter of auditory nerve fibers, whereas glycine and GABA, as well as glutamate, are associated with cochlear nucleus interneurons and non-auditory nerve innervations (Godfrey et al., 2000, 2008b). Among other amino acids, aspartate is closely related metabolically to glutamate (Dingledine and McBain, 1994; Ross et al., 1995) and may function as a neurotransmitter (Dingledine and McBain, 1994), including in the auditory nerve (Wenthold, 1978, 1979; Godfrey et al., 1988). Serine is a precursor for synthesis of glycine and glutamine for synthesis of glutamate (Dingledine and McBain, 1994; Ross et al., 1995). Taurine can act as an agonist at GABA and glycine receptors (Albrecht and Schousboe, 2005). Threonine and arginine are less closely related to amino acid neurotransmission but are components of proteins and active in various aspects of metabolism. Breakdown of threonine can lead to synthesis of glycine, and breakdown of arginine can lead to synthesis of glutamate, nitric oxide, or urea (Lehninger, 1975).

We have used microdissection combined with high performance liquid chromatography (HPLC) to map levels of glutamate, glycine, GABA, aspartate, glutamine, serine, taurine, threonine, and arginine within the cochlear nucleus of rats euthanized at different survival times after cochlear ablation. Cochlear ablation destroys the spiral ganglion containing the somata of the auditory nerve fibers, resulting in degeneration of the central processes of the auditory nerve. Measurements have been made within subparts of the anteroventral (AVCN), posteroventral (PVCN), and dorsal (DCN) subdivisions, as well as in the auditory nerve root (ANR), or interstitial nucleus (IN). We have also made some measurements in the output pathways of the cochlear nucleus, the trapezoid body and acoustic striae, and in the superior olivary complex.

A preliminary report of results from this study has been presented (Godfrey et al., 2004a). Some of the results for the PVCN were included in a previous publication (Godfrey et al., 2008a), but they are also included here for completeness.

The results are compared to those obtained previously for the other eighth cranial nerve system, the vestibular nuclear complex and its vestibular nerve root, after removal of its inner ear input by vestibular ganglionectomy (Li et al., 1996; Godfrey et al., 2004b).

2. Material and methods

All of the procedures used in this study have been described previously (Lowry and Passonneau, 1972; Godfrey and Matschinsky, 1976; Godfrey et al., 1988, 2000, 2004b, 2008a,b; Ross et al., 1995; Li et al., 1996; Jin et al., 2005).

2.1. Animals and cochlear ablations

The rat tissues used for this study had been prepared for previous studies of cochlear ablation effects on the cholinergic system (Jin et al., 2005; Jin and Godfrey, 2006). Rats were obtained from Harlan Sprague Dawley, Indianapolis, IN. Control rats received no surgery. In the lesioned rats, the left cochlea was ablated as described previously (Jin et al., 2005; Jin and Godfrey, 2006), and rats were euthanized at various times afterward. Samples were obtained from both sides of two control rats (one age-matched to the 7-day-survival rats and one to the 2-month-survival rats), one rat with 2 days survival after cochlear ablation, and three rats each with 7 days, 1 month (31–33 days), and 2 months (58–62 days) survival.

2.2. Isolation of tissue samples

Rats were euthanized by decapitation while deeply anesthetized with sodium pentobarbital (50–80 mg/kg) (Ovation Pharmaceutical), then their brains were isolated and frozen within 3–5 min of death in Freon (Fisher Friendly Freeze-It) chilled to its freezing point with liquid nitrogen. Frozen tissue blocks were stored at 80°C in double airtight containers until sectioning.

Transverse sections of frozen brains were cut 20 µm thick at 20°C in a cryostat. Except for some sections used for receptor binding studies (Jin and Godfrey, 2006), every third section was either placed into an aluminum rack (Lowry and Passonneau, 1972), mounted on a slide for staining for Nissl substance, or mounted on a slide for staining for either acetylcholinesterase or cytochrome oxidase activity. The aluminum racks were kept at –80°C, on blocks of dry ice placed in the cryostat, during sectioning and then put into a glass vacuum tube for freeze drying of the sections (Lowry and Passonneau, 1972). Freeze-dried sections were stored in the racks in the vacuum tube under vacuum in a freezer below 20°C.

Dissection of freeze-dried tissue into samples for assay was done at 25X magnification, in a room with relative humidity maintained at or below 50%. A drawing attachment on the Wild dissecting microscope was used to map sample locations and compare maps with nearby stained sections (Godfrey and Matschinsky, 1976). Samples were weighed on quartz-fiber microbalances (Lowry and Passonneau, 1972); most weighed between 0.1 and 1 µg. Samples were loaded into 300 µl-capacity glass tube inserts (from Waters Corporation, Milford, MA) for HPLC measurement of amino acids. Samples from comparable locations

in both cochlear nuclei of the same rat were included in the same assay, along with samples from a comparable location of a control rat cochlear nucleus.

2.3 Amino acid assay

For HPLC assay of free amino acid levels, 50% (vol/vol) methanol, containing thienyl-serine as an internal standard to correct for variations in injection volume, was added to the samples to extract the amino acids, then aliquots were withdrawn by a WISP autosampler and derivatized with ortho-phthaldialdehyde (from Sigma, St. Louis, MO, or Pierce, Rockford, IL). The fluorescent derivatized amino acids were separated by reversed-phase chromatography on a C8 column (Ace, from Mac-Mod Analytical, Chadds Ford, PA), using gradient elution. The eluate was passed through a Spectrovision fluorescence detector, and the peaks of fluorescence were quantitated via a computer using Millennium software (from Waters). Amino acids were identified by their retention times, and their levels were calculated by comparison to calibrated amino acid standard solutions included in the same assays (Hill et al., 1979; Ross et al., 1995; Godfrey et al., 2000, 2004b, 2008a). Standards were prepared from Standard H (from Pierce) supplemented with calibrated amounts of asparagine, glutamine, taurine, and GABA (from Sigma). Other reagents were from Sigma or from Fisher Scientific. Although measurements were made for 12 amino acids, data for asparagine, alanine, and tyrosine are not reported because measurements of their relatively low values were not sufficiently reliable in the assays for this study.

2.4 Tissue densities

During degeneration of a fiber tract after its transection, there is breakdown and removal of myelin from the tissue (McCaman and Robins, 1959; Franson and Ronnevi, 1984; Ludwin, 1990; George and Griffin, 1994). Our previous study suggested that this began within a month in the vestibular nerve root after vestibular ganglionectomy (Godfrey et al., 2004b). The density of freeze-dried brain tissue, as dry weight per volume, is strongly related to lipid content, particularly the lipid in myelin (Godfrey and Matschinsky, 1976). Since the levels of the amino acids are expressed per dry weight and since they are predominantly associated with the non-lipid portions of tissue, decreases in tissue density at later times after the transection of the auditory nerve during cochlear ablation could lead to apparent increases in amino acid levels. Measurements of tissue density at the different times after cochlear ablation were therefore made in the dissected sections to enable corrections for such apparent increases. Tissue density, as dry weight per volume, was determined by dividing the total dry weight of a region in each section (sum of sample weights) by the total volume of dissected tissue (total area of the dissected portion, measured in the traced section map, times the 20 μm section thickness). A source of error in these measurements is variation in actual section thickness among individual sections, and potentially even within the same section. We minimized any errors resulting from variations in thickness among sections by comparing dry weight per volume measurements for each region between the lesioned and control sides of the same section.

2.5 Data presentation

Levels of amino acids, as mmol/kg dry weight, were plotted onto the maps of the dissected sections. Also, the data for all samples within a defined region were averaged for each

group. Differences from control values were evaluated for statistical significance by analysis of variance and t-tests. Data for both sides of control rats were combined for these comparisons; there were no statistically significant differences between them. Based on the rationale for the Bonferroni correction (Pagano and Gauvreau, 1993), the significance level for considering a difference statistically significant in t-tests was made more stringent according to the number of comparisons to the control value.

2.6 Treatment of animals

Treatment of animals was approved by and in accordance with existing regulations of the University of Toledo Health Science Campus Institutional Animal Care and Use Committee, which are consistent with guidelines of the National Institutes of Health.

3. Results

3.1 Effects of cochlear ablation on tissue density

Changes in tissue density could affect interpretation of changes in amino acid levels. However, the only significant change in tissue density with time after cochlear ablation was for the interstitial nucleus at 2 months after surgery (Table 1). The validity of this difference, which suggests a decrease in lipid content in lesioned-side tissue (Godfrey and Matschinsky, 1976), was supported by measurement of the lipid proportion of dissected interstitial nucleus samples in one rat with 2 months survival after cochlear ablation. The lipid proportion on the lesioned side was 44.4%, less than the average for the contralateral samples: 63.2%, which was similar to the average value of 68 % measured previously for control rats (Godfrey et al., 2000).

Another factor that can affect interpretation of changes in amino acid levels is changes in regional volumes subsequent to auditory nerve fiber degeneration after cochlear ablation. As previously reported for the rats of this study (Jin and Godfrey, 2006), ratios between the areas of the same regions on both sides of the same sections suggest approximately 20% decreases in size of AVCN and PVCN, but no change in DCN size, on the lesioned side at 1–2 months after cochlear ablation.

The amino acid data were not recalculated to account for tissue density and volume changes after cochlear ablation, but these factors are considered for interpretation of the results.

3.2 Amino acid levels in the control rats

The amino acid levels for the control rats in this study were generally somewhat higher than those reported previously (Godfrey et al., 2000) but were similarly distributed among regions. Differences averaged across regions ranged from 56% higher for threonine to 17% higher for taurine. Arginine was the only amino acid with lower levels in this study: values averaged 35% lower than the previously reported values. These differences between the studies may be related to differences between the groups of rats and differences in the calibration plots for the HPLC standard solutions.

3.3 Effects of cochlear ablation on amino acid levels in the cochlear nucleus

Figures 1 and 2 show examples of microdissection maps, with data for glutamate and taurine in lesioned-side (left) and contralateral cochlear nuclei of one of the rats used in the study. For this rat, with 7 days survival after cochlear ablation, glutamate levels were profoundly reduced in the IN (I) and also clearly decreased in the PVCN (P), AVCN (A), the deep layer of the DCN (d), and the acoustic striae (S) on the left (lesioned) side, as compared to the right side. By contrast, for the same rat, taurine levels were increased on the lesioned side in these same regions.

Since, as in the sample maps (Figs. 1 and 2), the results were routinely quite consistent throughout each region, they are summarized on a regional basis for all the amino acids in all the rats in Tables 2 and 3. As shown in these tables, cochlear ablation resulted in decreased aspartate and glutamate levels, particularly at 2 and 7 days after surgery, in lesioned-side cochlear nucleus regions, particularly the IN and regions that receive relatively dense auditory nerve innervation: PVCN, AVCN, and deep layer of DCN (Harrison and Warr, 1962; Cohen et al., 1972; Lorente de Nó, 1981; Merchán et al., 1985; Potashner et al., 1997). Only rarely were there any statistically significant changes contralateral to the lesion: decreased glutamate in the DCN deep and molecular layers of the one rat with 2 days survival and decreases of both amino acids in the IN at 2 months survival. Overall, the lesioned-side changes can be most easily appreciated as the percentage differences between the two sides (Figure 3). The decreases for aspartate and glutamate were largest at the shorter survival times, perhaps partly because of the decreases in tissue density in the IN and volume within parts of the ventral cochlear nucleus (VCN) at longer survival times. At 2 days after cochlear ablation, there were unexpectedly sizeable lesioned-side decreases of aspartate in regions not receiving major auditory nerve innervation, particularly the DCN fusiform soma and molecular layers (Figure 3).

Curiously, there were some bilateral decreases of GABA level in the AVCN and PVCN, especially significant at 2 days survival in PVCN, which might result from general traumatic effects of the surgery (Demediuk et al., 1989; Godfrey et al., 2008a). Also curiously, there were statistically significant decreases of GABA level on the lesioned side, at 2 days - 1 month survival, in the DCN molecular layer, which receives relatively little innervation from the auditory nerve (Cohen et al., 1972; Lorente de Nó, 1981; Merchán et al., 1985).

There were relatively few changes in average glycine levels. These included some small ipsilateral increases and contralateral decreases in VCN regions and deep DCN at 2 days and at 2 months after cochlear ablation. The most consistent change was a small decrease in the contralateral fusiform soma layer at 2 days through one month.

All amino acids other than aspartate and glutamate tended to show increased ipsilateral-to-contralateral level ratios after cochlear ablation, especially in the ANR (IN) and regions receiving more substantial auditory nerve input: VCN and deep layer of DCN (Figure 3).

Although the rostral part of the PVCN was not systematically sampled, it was mapped in one rat with 7 days survival after left cochlear ablation. The maps in this rat, with results for glutamate, have been presented previously (Godfrey et al., 2008a). Average rostral PVCN

amino acid levels for this rat were similar to caudal PVCN levels except for taurine, which was 31 % higher in caudal PVCN on the left (lesioned) side ($P = 0.001$) and 37 % higher on the right side ($P < 0.001$). There was also a trend toward a difference for GABA, which was 26 % higher in caudal PVCN on the left side ($P = 0.02$) and 18 % higher on the right side ($P = 0.07$).

3.4 Effects on output pathways

A few measurements were made for the cochlear nucleus output pathways: the ventral acoustic stria, or trapezoid body, and the combined dorsal and intermediate acoustic striae.

For the trapezoid body, combined data for two lesioned rats, one with 7 days and one with 2 months survival, showed only one barely statistically significant ($P = 0.04$) difference between left and right sides: the average taurine level was 11 % higher in the left (lesioned-side) trapezoid body.

For the acoustic striae, data were obtained from two rats with 7 days survival, including that shown in Figures 1 and 2, and one rat each with 2 days, 1 month, and 2 months survival. At each survival time, aspartate and glutamate levels were lower, and serine, glutamine, and taurine levels higher on the lesioned side compared to contralateral. When data for all the survival times were combined, statistically significant differences included aspartate 18% lower ($P = 0.01$), glutamate 21% lower ($P = 0.0004$), serine 21% higher ($P = 0.02$), and glutamine 12% higher ($P = 0.02$) on the lesioned side. The difference for taurine, 15% higher on the lesioned side, barely missed statistical significance ($P = 0.05$). Although some studies have not shown auditory nerve fibers penetrating the acoustic striae (e.g., Lorente de Nó, 1981, Fig. 6-1), some auditory nerve fibers have been observed in the acoustic striae region of rats (M. A. Merchán, personal communication). Another possibility is that these changes in amino acid levels represent transneuronal effects on acoustic striae axons of DCN and PVCN neurons, but such effects were not observed in the trapezoid body except for taurine.

3.5 Effects on superior olivary regions

Measurements were made for superior olivary nuclei in one lesioned rat at each survival time: 2 days, 7 days, 1 month, and 2 months. Samples of the lateral superior olivary nucleus (LSO) and medial nucleus of the trapezoid body (MNTB) were obtained from all four rats, whereas samples for the superior paraolivary nucleus, ventral nucleus of the trapezoid body, and periolivary regions were obtained only for the rats with 7 days and 2 months survival. No statistically significant difference, at $P < 0.05$, between left and right sides was found for any amino acid in any region except for GABA in the LSO. The average level of GABA was lower in the left (lesioned side) LSO at 7 days, 1 month, and 2 months after cochlear ablation, and throughout the medial-lateral extent of the LSO (Fig. 4).

4. Discussion

4.1 Cochlear ablation effects on amino acid levels in rats and comparison to effects of cochlear ablation on markers of amino acid neurotransmission in guinea pigs

Our results for glutamate and aspartate in rat cochlear nucleus regions at 2 and 7 days after cochlear ablation resemble those previously reported for guinea pig at 2 and 7 days after cochlear ablation (Wentholt, 1978) in that lesioned-side levels, expressed as percentages of contralateral values, decreased by similarly very large amounts in the IN (ANR), and also by large amounts in PVCN and AVCN. There are a few differences: the effects on aspartate and glutamate were similar in rat cochlear nucleus, whereas in guinea pig the effects on aspartate were generally larger than those on glutamate, and the effects on both amino acids in the DCN deep layer were larger and those on aspartate in the AVCN smaller in rat than in guinea pig.

Our results for glutamate and aspartate are in some ways consistent with previous reports of changes in release and uptake of D-aspartate, as representative of synaptic release and uptake of glutamate, in guinea pigs (Potashner et al., 1997), in that decreases were found in AVCN, PVCN, and DCN after cochlear ablation. However, the release and uptake measurements showed more contralateral changes than our measurements of glutamate and aspartate levels and larger relative ipsilateral changes for release, especially at 2 days after cochlear ablation. Also, a relatively large decrease in D-aspartate release in ipsilateral DCN at 2 days after cochlear ablation was maintained through 145 days, whereas decreases in glutamate and aspartate levels in all ipsilateral DCN layers 2 days after cochlear ablation showed recovery at later times, especially in the fusiform soma and molecular layers.

The decreased glutamate levels in AVCN main, PVCN caudal, and DCN deep layer, but not in DCN molecular layer, ipsilateral to the cochlear ablation, as compared to contralateral (Figure 3), resemble previously reported decreases of binding of 6-cyano-7-nitro-quinoxaline-2,3-dione (CNQX) to α -amino-3-hydroxy-5-methyl-isoxazole-4-propionate (AMPA)-type glutamate receptors in AVCN, PVCN, and deeper layers of DCN, but not in DCN molecular layer, after cochlear ablation in rats (Li et al., 1997). Such differences between ipsilateral and contralateral cochlear nucleus regions were not so evident in a study of AMPA binding in guinea pigs, but the authors mentioned that the AMPA at the concentration used would likely bind to intracellular AMPA receptors, whereas the CNQX would likely bind to membrane receptors (Suneja et al., 2000).

The generally small effects of cochlear ablation on GABA levels in cochlear nucleus regions (Table 2) are consistent with previous findings of minimal effects of bilateral cochlear ablation on measures of GABA uptake and release (Potashner et al., 1985). Most of the GABA in the cochlear nucleus appears related to intrinsic neurons and centrifugal innervation (Fisher and Davies, 1976; Potashner et al., 1985; Godfrey et al., 1988, 2000; Shannon-Hartman et al., 1993; Suneja et al., 1998b).

The unexpected 20–30 % decreases of the average GABA level in the lesioned-side DCN molecular layer after cochlear ablation must represent an indirect effect since auditory nerve fibers contain little if any GABA and provide very little innervation of the DCN molecular

layer (Cohen et al., 1972; Lorente de Nó, 1981; Merchán et al., 1985). The relatively high GABA levels in this region appear to be contained mostly in axons and terminals of its centrifugal innervation (Godfrey et al., 1988; Shannon-Hartman et al., 1993) and in GABA- and glutamic acid decarboxylase-immunopositive interneurons, including especially stellate and cartwheel cells (Wouterlood and Mugnaini, 1984; Mugnaini, 1985; Alibardi, 2003). Since the GABA decreases are present even at 2 days after cochlear ablation, changes in the centrifugal innervation seem less likely than changes in the local interneurons. Changes in interneurons might result from lesion-induced changes in neurons synapsing with them, such as granule cells or neurons of the DCN deep layer, retrograde effects because of changes in neurons that they innervate, or trauma-related effects, as mentioned concerning GABA changes in the VCN. It may be noted that there were also consistent, although not statistically significant at any one survival time, lesioned-side decreases of average GABA level in the DCN fusiform soma layer, which also contains many cartwheel cells (Wouterlood and Mugnaini, 1984), and that cartwheel cells in many respects resemble cerebellar Purkinje cells (Wouterlood and Mugnaini, 1984; Mugnaini, 1985; Berrebi and Mugnaini, 1991), which appear to be unusually vulnerable to indirect effects of traumatic injury (Potts et al., 2009). Also, since cartwheel cells provide innervation to fusiform cells (Berrebi and Mugnaini, 1991), there could be a retrograde effect upon cartwheel cell activity because of changes in fusiform cells after loss of their auditory nerve innervation.

Small increases of glycine level, particularly ipsilaterally in the IN and PVCN, after cochlear ablation in our study can be compared to more prominent measured increases of glycine uptake and decreases of glycine release, especially in the DCN (Suneja et al., 1998b; Potashner et al., 2000), and decreases of glycine receptor binding in most cochlear nucleus regions up through 60 days after cochlear ablation (Suneja et al., 1998a; Potashner et al., 2000). The small decreases of average glycine level in the fusiform soma layer, statistically significant contralateral to the lesion, may correlate with the small decreases of average GABA level in that same layer, which were consistent across time although not statistically significant at any one time point. This correlation may support a cochlear ablation effect on cartwheel cells, as mentioned above, since cartwheel cells appear to contain both glycine and GABA (Wentholt et al., 1987; Apostolides and Trussell, 2013).

4.2 Comparison of cochlear ablation effects on amino acid levels to those on choline acetyltransferase (ChAT) activity

The rats used for this study were the same ones as for studies of the effects of cochlear ablation on ChAT activity and muscarinic acetylcholine receptor binding in the cochlear nucleus (Jin et al., 2005; Jin and Godfrey, 2006). Increases of ChAT activity, especially at one month after cochlear ablation, were measured in the lesioned-side AVCN and PVCN, some of which were accounted for by decreases in size of these regions by a month after cochlear ablation (Jin et al., 2005). As much as 25 % of the increases in levels of amino acids other than aspartate and glutamate, and reversals of the decreases in aspartate and glutamate levels, 1–2 months after cochlear ablation, in AVCN main part, caudal PVCN, and IN can be related to shrinkage of those regions after the lesion. (Although area ratios could not be estimated for IN because of variations in the location of auditory nerve transection during brain removal, we assume that shrinkage of this auditory nerve fiber-rich region after

cochlear ablation should be at least as much as that of AVCN and PVCN, and a 23 % decrease in tissue density at 2 months was estimated, consistent with loss of myelin.)

The decreases of GABA levels in the lesioned-side LSO, as compared to contralateral, resemble those for ChAT activities (Jin et al., 2005) and suggest that, as for ChAT, they represent a retrograde effect of damage to the olivocochlear fibers. Although levels of GABA and activities of its synthetic enzyme glutamate decarboxylase are very low in the cochlear hair cell regions (Tachibana and Kuriyama, 1974; Godfrey et al., 1976; Fex and Wenthold, 1976), and GABA levels are low in the LSO (Godfrey et al., 2000), this result, together with evidence from several other studies (Richrath et al., 1974; Drescher et al., 1983; Bobbin et al., 1990; Vetter et al., 1991; Eybalin, 1993; Jenkins and Simmons, 2006), suggests an association of GABA with some lateral olivocochlear neurons. However, whereas immunohistochemical results have suggested that GABAergic olivocochlear neuron somata are preferentially located in the lateral part of the LSO in both rat (Vetter et al., 1991) and hamster (Jenkins and Simmons, 2006), our measurements showed higher GABA levels medially. Also, whereas immunohistochemical results have suggested separate populations of GABAergic and cholinergic olivocochlear neurons (Vetter et al., 1991), our measurements are consistent with all olivocochlear neurons being cholinergic (Godfrey et al., 1984), so that GABA would be colocalized in some lateral olivocochlear neurons, as may be the case in some skeletal motoneurons (Chan-Palay et al., 1982).

4.3 Comparison of cochlear ablation effects on amino acid levels in rat auditory nerve root and cochlear nucleus to vestibular ganglionectomy effects on rat vestibular nerve root and vestibular nuclei

The early portions of the auditory and vestibular systems are closely related with respect to the locations of their receptors in the inner ear, connections to the brain via the eighth cranial nerve, and locations of their first brain nuclei near the ponto-medullary junction. Effects of destruction of the somata of their eighth nerve innervations on amino acid levels in rats can be directly compared since the same methods of analysis were used in this study of the auditory system and our previous studies of the vestibular system (Li et al., 1996; Godfrey et al., 2004b).

First, results for the IN, or ANR, are compared with those for the vestibular nerve root (VNR). Destruction of the somata of the neurons contained in the nerve roots should be equivalent to axotomy and is expected to lead to degenerative processes, including breakdown of axoplasm, breakdown and removal of myelin, and glial infiltration, especially by microglia (McCaman and Robins, 1959; Franson and Ronnevi, 1984; Ludwin, 1990; George and Griffin, 1994; Aldskogius and Kozlova, 1998). Myelin loss, which is expressed in our studies as decreased dry weight per volume and decreased lipid content of tissue, was more rapid in the VNR, where it was detectable even by 2 weeks after ganglion destruction (Godfrey et al., 2004b), than in the ANR, where it was not detectable until 2 months after cochlear ablation (Table 1). The reasons for this difference are not clear. The vestibular nerve contains a more heterogeneous population of fiber diameters than the auditory nerve (Walsh et al., 1972), with most fiber diameters in the guinea pig smaller in the vestibular than in the auditory nerve (Gacek and Rasmussen, 1961), but there appears to be no clear

relationship between fiber diameter and rate of degeneration after axotomy (Guillery and Taylor, 1993). Degenerative processes have been found to progress faster closer to the site of axotomy than farther from it (Guillery and Taylor, 1993), but the sampling location for the VNR in our studies was probably no closer to the site of axotomy than that for the ANR. Glutamate and aspartate levels decreased on the lesioned-side in both the ANR and VNR, but, unlike in the ANR, the levels increased in the contralateral VNR at shorter survival times (Godfrey et al., 2004b). This resulted in the lesioned-side changes, when expressed as a percentage of contralateral values, being somewhat larger in the VNR than in the ANR at shorter survival times, whereas they were similar one month after the lesion. In both nerve roots, the levels of aspartate and glutamate decreased contralaterally at the longest survival time studied relative to the preceding sampling time. The lesioned-side changes, as percentages of contralateral values, for the other amino acids, as well as for glutamate and aspartate, are compared between ANR and VNR in Figure 5. For the amino acids other than glutamate and aspartate, results for the ANR and VNR were quite different at 1 week after lesion, in that the levels in the lesioned-side VNR were lower than contralateral values, whereas those in the lesioned-side ANR were similar to or higher. By a month after lesion, results for the two nerve roots became similar, in that lesioned-side levels were consistently higher than contralateral values for all amino acids except glutamate and aspartate. The lesioned-side relative increases in the VNR could be partially related to its decreased tissue density because of myelin loss, but in many cases they reflect decreased amino acid levels in the contralateral VNR (Godfrey et al., 2004b); lesioned-side increases in both nerve roots could also be related to glial infiltration.

Post-lesion amino acid level changes in cochlear nucleus regions, expressed as percentages of contralateral values (Fig. 3), can be compared to those for vestibular nuclei regions, expressed in the same way (Li et al., 1996, Fig. 1). Aspartate and glutamate level decreases on the lesioned side in caudal PVCN were larger than in any of the vestibular nuclear regions, but the decreases in the AVCN main part and deep DCN were of similar magnitude as in most of the vestibular nuclear regions. The magnitudes of the decreases in the rest of the cochlear nucleus regions more closely resembled the smaller changes in the dorsal part of the lateral vestibular nucleus and the ventral part of the medial vestibular nucleus. The trend for level differences between the two sides to become less by 1 month after the lesion, found in all cochlear nucleus regions except caudal PVCN, was observed in the superior and lateral vestibular nuclei but not in the spinal or medial nuclei. As in the cochlear nucleus regions, glutamine and taurine levels increased in most lesioned-side vestibular nuclear regions as compared to contralateral. Since these two amino acids tend to be more concentrated in glia than in neurons (Patel and Hunt, 1985; Ottersen et al., 1992; Hassel et al., 1995), their increases may be related to the gliosis reported in both cochlear and vestibular nuclei after inner ear lesions (Campos Torres et al., 1999; Li et al., 1999; Fuentes-Santamaría et al., 2012). On the other hand, the increases in glutamine and taurine levels after the peripheral lesion were generally less striking in vestibular nuclei than in the cochlear nucleus, as were any changes in the levels of the other amino acids less closely related to synaptic transmission. The increases of these amino acid levels in the cochlear nucleus seem related to the degeneration of the auditory nerve fibers because their magnitudes at least roughly correlate with the relative densities of auditory nerve fibers and

terminals in the various cochlear nucleus regions. Besides any effects from gliosis, these increases may result from protein breakdown, resulting in release of free amino acids, and changes in metabolism of surviving cells, particularly neurons that have lost much of their innervation.

4.4 Comparison of cochlear ablation effects on amino acid levels in rat cochlear nucleus with intense sound effects on amino acid levels in hamster cochlear nucleus

Whereas cochlear ablation leads to degeneration of all auditory nerve fibers, other types of cochlear damage lead to degeneration of just some fibers. The cochlear damage that results from exposure to intense sound leads to degeneration of relatively small numbers of auditory nerve fibers (Morest et al., 1998; Kim et al., 2004) compared to cochlear ablation. Likewise, the effects of intense sound on amino acid levels in the cochlear nucleus, based on our studies in hamsters (Godfrey et al., 2008b, 2012), are much less than those of cochlear ablation. Also, the changes measured are often in opposite directions. Thus, the decreased glutamate and aspartate levels in cochlear nucleus regions after cochlear ablation contrast with a tendency toward increased glutamate and aspartate levels after intense tone exposure (Godfrey et al., 2012). The trend for levels of the other amino acids to increase in cochlear nucleus regions after cochlear ablation was not found after intense tone exposure. Rather, there were some trends toward decreased levels, particularly for taurine (Godfrey et al., 2012).

4.5 Conclusions

Our results are consistent with much other evidence that glutamate is a neurotransmitter of auditory nerve fibers (Wentholt and Gulley, 1977; Wentholt, 1978, 1979; Wentholt et al., 1993; Godfrey et al., 1988; 2000; 2008a; Hackney et al., 1996; Potashner et al., 1997; Suneja et al., 2000). They also continue evidence for association of aspartate with auditory nerve fibers (Wentholt and Gulley, 1977; Wentholt, 1978, 1979; Godfrey et al., 2008a), perhaps through its close metabolic relationship with glutamate (Dingledine and McBain, 1994; Ross et al., 1995).

The increases of other amino acids in cochlear nucleus regions after cochlear ablation are consistent, as for ChAT, with their having no major association with auditory nerve fibers.

Effects of cochlear ablation on amino acid levels in the auditory nerve root and cochlear nucleus show many parallels to those of vestibular ganglionectomy on amino acid levels in the vestibular nerve root and vestibular nuclei, but there are also some differences.

The changes of amino acid levels in cochlear nucleus regions are much larger after cochlear ablation than after intense tone exposure and often in the opposite direction.

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ABBREVIATIONS

AMPA α -amino-3-hydroxy-5-methyl-isoxazole-4-propionate

ANR	auditory nerve root
AVCN	anteroventral cochlear nucleus
ChAT	choline acetyltransferase
CNQX	6-cyano-7-nitro-quinoxaline-2,3-dione
DCN	dorsal cochlear nucleus
GABA	γ -aminobutyric acid or γ -aminobutyrate
HPLC	high performance liquid chromatography
IN	interstitial nucleus (auditory nerve root)
LSO	lateral superior olivary nucleus
MNTB	medial nucleus of the trapezoid body
PVCN	posteroventral cochlear nucleus
VCN	ventral cochlear nucleus
VNR	vestibular nerve root

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Highlights

Glutamate concentrations decrease in cochlear nucleus regions after cochlear ablation

Aspartate concentrations decrease in cochlear nucleus regions after cochlear ablation

Taurine concentrations increase in cochlear nucleus regions after cochlear ablation

Effects of eighth nerve damage show similarities in auditory and vestibular systems

Cochlear ablation affects cochlear nucleus chemistry differently than intense sound

Figure 1.

Maps of microdissected freeze-dried transverse sections for lesioned-side (left side) and contralateral (right side) cochlear nuclei of a rat with 7 days survival after left cochlear ablation, with glutamate levels in mmol/kg dry weight plotted onto sample locations. Section numbers are presented at bottom; lower numbers are more caudal. In the maps, thick solid lines are outside borders of the freeze-dried sections, thick dashed lines are inside regional boundaries seen in the freeze-dried sections, dotted lines are regional boundaries traced from adjacent stained sections, and thin solid lines are tracings of the cuts made to isolate samples for assay. Scale bar at right shows dorsal (D), ventral (V), lateral (L), and medial (M) directions. Regional abbreviations are A, anteroventral cochlear nucleus (AVCN); G, granular region; I, interstitial nucleus (IN, or auditory nerve root); P, posteroventral cochlear nucleus (PVCN); d, f, and m, deep, fusiform soma, and molecular layers of dorsal cochlear nucleus.

Figure 2.

Maps of the same microdissected freeze-dried transverse sections as in Figure 1, with taurine levels in mmol/kg dry weight plotted onto sample locations. Details and abbreviations as in Figure 1.

Figure 3. Comparisons between lesioned (left) and unlesioned (right) sides of amino acid levels in cochlear nucleus regions of rats with cochlear ablations and various survival times. Lesioned-side level minus unlesioned side level is expressed as percentage of the unlesioned-side (contralateral) level in each case. Standard errors and statistically significant differences are shown in Tables 2 and 3, and the significant differences between the two sides are repeated here: * $p < 0.01$, ** $p < 0.001$. Some of the bars for IN (ANR) were allowed to go off scale in order to keep the same scale as for the other plots. Exact values can be calculated from the tables.

Figure 4.

Plots of amino acid levels (mean $\pm$ SEM) vs. post-surgical survival time and lateral-to-medial position in the lateral superior olivary nucleus (LSO) on each side of one rat at 2 days, 7 days, 1 month (31 days), and 2 months (62 days) after left cochlear ablation surgery. For each LSO, 4 samples were dissected, so that $n = 4$ for each data point in the plots of the left column. Since the LSO was not dissected into lateral-to-medial sample locations for the left side of the rat with 1 month survival, the data points for the plots in the right column represent the averages for the rats with 2 days, 7 days, and 2 months survival ($n = 3$). The differences in GABA level between left (lesioned side) and right (contralateral) LSO were statistically significant at 7 days ($P = 0.036$), 1 month ($P = 0.002$), and 2 months ($P = 0.014$), and in positions 2 (second from lateralmost, $P = 0.032$) and 4 (medialmost, $P = 0.025$). For all samples combined ($n = 16$), the GABA level difference between left and right LSO was significant at $P = 0.000008$.

Figure 5.

Comparison of effect of nerve transection on amino acid levels in the central portions (nerve roots) of the auditory and vestibular nerves. The data for the auditory nerve root (or interstitial nucleus) are from the present study, in which the nerve was transected by the cochlear ablation, while those for the vestibular nerve root are from a previous study (Godfrey et al., 2004b), in which the nerve was transected by vestibular ganglionectomy. Percentage differences between lesioned-side and contralateral nerve roots are compared at 1 week and 1 month after surgery, the two survival times that match between the studies. Because of the very low concentrations of GABA in the nerve roots, the sizeable percentage changes correspond to only small mmol/kg dry wt changes: -0.1 and $+0.2$ for ANR and -0.4 and $+1.0$ for VNR at 1 week and 1 month, respectively.

Table 1Left/right ratios of tissue densities in rat cochlear nucleus at different times after left cochlear ablation^a

Region	Control	2–7 days	1 month	2 months
AVCN main part	1.05	1.05 ± 0.04	0.93 ± 0.02	1.00 ± 0.04
IN (ANR)	1.02	1.10 ± 0.20	0.97 ± 0.08	0.77 ± 0.06
PVCN caudal part	1.12	1.00 ± 0.13	0.94 ± 0.08	0.92 ± 0.09
DCN deep layer	1.01	0.91 ± 0.02	1.09 ± 0.06	0.99 ± 0.07
DCN fusiform soma layer	1.10	0.96 ± 0.03	1.03 ± 0.04	0.96 ± 0.03
DCN molecular layer	1.01	0.98 ± 0.06	0.95 ± 0.02	1.02 ± 0.07

^aData are presented as mean for n = 2 control rats and mean ± SEM for n = 4 rats with 2–7 day survival and 3 each with 1 and 2 months survival. The only statistically significant difference between exposed and control is for IN at 2 months vs. all other times combined (P = 0.03). Abbreviations are AVCN, anteroventral cochlear nucleus; IN, interstitial nucleus; PVCN, posteroventral cochlear nucleus; and DCN, dorsal cochlear nucleus.

Table 2

Concentrations of transmitter-related amino acids in rat cochlear nucleus at different times after cochlear ablation^a

Region	Control	2 days		7 days		1 month		2 months	
		L	R	L	R	L	R	L	R
<u>Aspartate</u>									
AVCN granular	13.6±0.9	13.3	19.3±2.3	13.9±0.5	15.3±0.9	14.8±1.7	16.5±1.1	11.8±0.8	13.0±0.8
AVCN subgranular	18.8±0.3		19.6	15.7±0.5 ^{cx}	19.1±0.1	19.7±1.9	21.6±1.5	15.6±1.2	17.7±0.8
AVCN main	17.5±0.6	12.4±1.6	18.2±1.2	14.1±0.6 ^{dy}	17.4±0.6	17.6±1.0	18.0±1.0	14.4±0.9	15.4±0.5
IN (ANR)	7.5±0.7	4.4	8.5±0.3	2.9±0.4 ^{dy}	6.5±0.6	5.0±1.0	6.4±0.3	4.7±0.3 ^c	5.0±0.3 ^c
PVCN caudal	13.1±0.7	9.7±0.2 ^{dx}	14.9±1.3	9.1±0.3 ^{dy}	14.4±0.5	9.0±0.5 ^{dy}	13.0±0.3	10.8±0.6	12.3±0.4
DCN deep	14.7±0.5	10.5±0.8 ^d	12.8±0.6	12.4±0.7 ^x	15.9±0.7	15.1±0.7	16.2±0.6	14.1±0.6	15.6±0.8
DCN fusiform soma	17.7±0.4	11.7±0.6 ^{dy}	16.5±0.9	16.0±0.6 ^x	18.6±0.4	16.9±0.4	18.0±0.6	17.1±0.6	17.5±0.5
DCN molecular	14.0±0.4	9.5±0.6 ^{dy}	12.8±0.5	14.0±0.6	15.4±0.4	13.7±0.2	14.2±0.4	14.3±0.6	14.7±0.4
<u>Glutamate</u>									
AVCN granular	47.4±2.9	43.6	47.3±2.4	39.0±1.2	44.5±0.9	45.9±2.7	45.9±2.0	41.5±1.0	45.9±1.2
AVCN subgranular	37.4±1.7		42.7	29.2±1.5 ^x	37.9±0.4	35.7±1.4	36.0±0.9	36.0±2.0	37.3±1.2
AVCN main	31.9±0.7	24.1±2.6	32.2±1.3	23.6±0.8 ^{dy}	33.0±0.7	27.6±1.4	31.7±1.2	29.3±1.6	31.6±1.2
IN (ANR)	19.7±1.4	9.0	19.6±0.9	8.0±0.8 ^{dy}	16.8±1.3	12.7±1.8	17.1±0.7	10.8±0.8 ^{dx}	14.8±0.9 ^c
PVCN caudal	27.8±1.0	17.5±0.5 ^{dy}	27.7±1.5	18.9±0.6 ^{dy}	29.4±0.8	17.7±0.7 ^{dy}	25.9±0.5	20.8±0.9 ^{dy}	25.5±0.7
DCN deep	33.9±0.8	23.4±1.9 ^d	29.7±0.7 ^c	27.6±1.5 ^{cx}	34.5±1.1	28.8±1.9	32.7±0.8	29.2±0.7 ^d	33.5±1.2
DCN fusiform soma	45.1±1.1	37.2±1.7 ^c	41.0±1.3	41.6±0.9	45.6±1.4	39.7±1.8	42.5±1.2	42.4±1.0	42.8±1.0
DCN molecular	58.7±1.1	43.1±3.0 ^c	46.7±1.1 ^d	55.6±1.5	57.4±1.4	55.1±2.9	54.4±1.7	58.7±1.3	56.4±1.7
<u>GABA</u>									
AVCN granular	9.1±0.6	7.3	7.9±0.4	8.7±0.2	9.0±0.5	7.9±0.6	8.6±0.1	8.6±1.2	7.9±0.5
AVCN subgranular	9.6±1.0		8.0	8.8±0.6	9.0±0.6	8.2±0.5	7.2±0.2	8.4±0.4	8.0±0.6
AVCN main	7.1±0.4	5.4±0.5	5.7±0.3	6.1±0.2	6.1±0.3	5.9±0.4	5.3±0.2 ^c	6.0±0.3	5.4±0.3 ^c
IN (ANR)	1.2±0.2	2.0	1.3±0.2	1.2±0.1	1.3±0.2	1.2±0.1	1.0±0.2	1.3±0.3	0.6±0.1
PVCN caudal	4.3±0.3	2.9±0.2 ^d	3.0±0.2 ^d	4.5±0.2	4.8±0.2	4.0±0.2	3.7±0.1	5.1±0.2 ^y	3.6±0.2
DCN deep	6.2±0.4	5.4±0.4	5.9±0.3	6.2±0.2	6.4±0.4	6.2±0.3	5.6±0.1	6.9±0.4	6.1±0.2

Region	Control	2 days		7 days		1 month		2 months	
		L	R	L	R	L	R	L	R
DCN fusiform soma	11.2±0.8	8.8±0.2	9.2±0.5	9.6±0.3	10.6±0.6	9.0±0.3	9.9±0.5	10.1±0.3	10.3±0.4
DCN molecular	13.7±0.7	9.2±0.3 ^d	10.9±0.3 ^c	11.1±0.5 ^c	13.4±0.5	11.0±0.3 ^c	12.0±0.2	12.3±0.3	13.2±0.2
<u>Glycine</u>									
AVCN granular	26.1±0.6	34.8	31.0±2.8	32.1±3.0	28.7±2.0	30.2±1.8	28.8±1.0	23.4±1.1	26.1±1.3
AVCN subgranular	29.5±0.7		28.6	30.4±2.4	28.8±0.9	30.2±0.6	27.0±0.6	28.5±1.4	25.5±0.4 ^c
AVCN main	25.3±0.8	29.6±1.5 ^x	23.2±1.3	25.7±0.7	23.0±0.9	25.0±0.8	22.4±0.8	24.4±0.9	21.7±0.7 ^c
IN (ANR)	7.5±0.6	12.1	10.3±0.8	8.4±0.6	8.7±1.0	8.6±0.4	7.0±0.5	10.1±0.5 ^c	5.8±0.4
PVCN caudal	16.8±0.8	19.8±0.7 ^y	14.1±0.5	18.4±0.8	17.5±0.7	18.1±0.9	16.7±0.6	21.7±1.0 ^c	15.3±0.4
DCN deep	28.9±1.7	26.5±1.2	22.8±0.7 ^c	27.4±1.0	25.9±1.2	28.7±0.5	25.6±1.3	28.4±0.6	26.2±1.1
DCN fusiform soma	32.9±0.7	30.6±1.4	27.4±0.9 ^d	30.4±0.7	29.1±0.8 ^c	30.1±0.9	29.3±0.8 ^c	31.4±0.4	30.7±0.5
DCN molecular	32.0±0.7	30.7±2.2	29.3±0.8	30.5±0.9	31.4±0.9	29.3±1.3	29.6±0.8	31.8±0.6	31.9±0.5

^aData are mmol/kg dry wt, presented as mean ± SEM, based on numbers of samples from 2 control rats and 10 lesioned rats: one 2-day and 3 each of 7-day, 1-month, and 2-month post-surgery survivals. Abbreviations are: ANR, auditory nerve root; AVCN, anteroventral cochlear nucleus; DCN, dorsal cochlear nucleus; PVCN, posteroventral cochlear nucleus. Numbers of samples assayed for control rats and for lesioned rats, left and right sides at each survival time, in order of presentation, are: AVCN granular 4, 0, 1, 4, 3, 4, 5, 4, 5; AVCN main 12, 7, 9, 12, 16, 13, 16, 11, 13; IN 11, 2, 3, 14, 12, 5, 5, 6, 10; PVCN caudal 16, 4, 10, 21, 25, 15, 21, 10, 17; DCN deep 11, 8, 9, 10, 12, 9, 11, 9, 9; DCN fusiform soma 11, 7, 9, 11, 9, 9; DCN molecular 11, 7, 9, 10, 9, 11, 9, 10. In one case for glutamate and in 8 cases for aspartate and glycine, the number of samples was 1 less, and in one case for aspartate and glycine, 2 less, because of contamination. Statistical significances of differences between exposed and control are

^c $P < 0.01$ and

^d $P < 0.001$, and between left and right sides of exposed

^x $P < 0.01$ and

^y $P < 0.001$.

Table 3Concentrations of other amino acids in rat cochlear nucleus at different times after cochlear ablation^a

Region	Control	2 days		7 days		1 month		2 months	
		L	R	L	R	L	R	L	R
Glutamine									
AVCN granular	30.6±1.1	28.3	30.4±1.9	29.8±1.4	30.1±1.2	32.6±0.8	29.5±1.2	30.6±1.6	30.4±1.8
AVCN subgranular	24.9±1.4		26.4	23.6±0.4	22.0±0.9	26.4±1.0 ^x	21.3±0.4	27.3±1.3	25.1±1.1
AVCN main	20.9±0.4	22.2±1.0 ^x	18.3±0.6 ^c	19.7±0.4	18.9±0.6 ^c	22.1±0.7 ^y	18.6±0.5 ^c	23.4±1.2	20.9±1.2
IN (ANR)	12.1±0.7	14.5	12.2±0.8	11.9±0.7	11.2±0.6	13.0±0.6	11.0±0.4	15.5±1.3 ^x	10.2±0.5
PVCN caudal	19.3±0.9	19.2±0.9	15.3±0.8 ^c	19.6±0.9	18.6±0.6	21.5±1.7	19.0±0.7	26.0±1.4 ^{dy}	19.0±0.7
DCN deep	19.7±0.6	20.4±1.1	17.3±0.5 ^c	20.0±0.7	18.1±0.6	22.9±0.5 ^{dy}	18.9±0.6	23.9±0.9 ^c	20.7±1.2
DCN fusiform soma	27.1±0.5	26.5±0.9 ^x	23.2±0.6 ^d	25.6±0.5	24.2±0.7 ^c	28.3±1.0	25.1±0.7	30.2±1.1	26.7±0.9
DCN molecular	31.3±0.6	28.0±1.4	28.5±0.5 ^c	29.6±0.8	29.3±0.7	33.2±1.3	30.2±0.8	36.2±1.3 ^c	33.2±1.1
Taurine									
AVCN granular	20.0±1.7	16.4	17.1±2.5	20.7±2.6	19.0±0.8	19.7±1.1	17.3±1.3	17.6±0.8	18.1±1.5
AVCN subgranular	12.6±0.7		14.4	13.4±0.9	12.0±0.7	12.9±0.7 ^x	9.5±0.3	14.0±0.8	10.9±0.5
AVCN main	9.2±0.2	11.0±0.9	8.3±0.6	10.5±0.5	9.6±0.5	10.5±0.4 ^{cy}	7.9±0.2 ^d	11.5±0.6 ^{cx}	8.9±0.5
IN (ANR)	5.9±0.2	14.3	5.4±0.8	9.5±0.5 ^{dy}	6.8±0.3	10.6±1.8	6.0±0.5	12.4±0.8 ^{dy}	5.4±0.2
PVCN caudal	10.4±0.5	12.7±1.2	7.2±0.6 ^d	10.9±0.4	9.8±0.3	12.8±0.8 ^y	9.1±0.3	16.7±0.7 ^{dy}	9.3±0.3
DCN deep	8.6±0.2	9.9±0.5	8.1±0.5	8.7±0.4	8.0±0.5	9.4±0.3 ^x	8.3±0.2	10.7±0.5 ^x	9.0±0.2
DCN fusiform soma	12.3±0.3	14.7±0.7	11.9±1.0	10.9±0.4	11.3±0.9	11.7±0.3	11.2±0.2	13.3±0.2 ^y	11.9±0.2
DCN molecular	19.6±0.4	21.4±0.6	18.2±1.0	17.3±1.2	17.9±1.3	18.4±0.6	17.6±0.5 ^c	21.0±0.4	19.7±0.5
Serine									
AVCN granular	8.0±0.6	11.5	13.2±0.7 ^c	9.8±1.0	9.3±0.8	9.4±0.4	7.9±0.7	9.2±0.9	10.2±1.0
AVCN subgranular	5.9±0.5		8.7	6.7±0.5	6.3±0.2	7.1±0.6	4.8±0.2	6.8±0.6	6.4±0.7
AVCN main	4.5±0.2	6.9±0.4 ^d	6.2±0.4 ^d	5.6±0.2 ^c	4.7±0.3	5.5±0.2 ^{cx}	4.2±0.3	5.9±0.5	4.5±0.3
IN (ANR)	2.8±0.2	3.6	3.9±0.2 ^c	4.3±0.5 ^c	4.4±0.5	3.9±0.3 ^c	3.2±0.4	5.3±0.6 ^{cx}	2.8±0.3
PVCN caudal	4.7±0.4	7.8±0.6 ^{cx}	4.7±0.3	6.8±0.6 ^c	5.7±0.4	6.7±0.6 ^c	5.4±0.4	6.8±0.7	4.7±0.3
DCN deep	4.7±0.3	4.6±0.6	3.7±0.2	5.8±0.4	4.6±0.2	5.9±0.4	4.4±0.2	5.2±0.4	5.0±0.5
DCN fusiform soma	5.4±0.2	4.4±0.3	5.3±0.5	6.1±0.3	5.7±0.2	6.3±0.4	5.4±0.5	6.0±0.5	5.7±0.4

Region	Control	2 days		7 days		1 month		2 months	
		L	R	L	R	L	R	L	R
DCN molecular	7.6±0.4	6.6±0.7	7.1±0.6	8.5±0.3	8.7±0.4	8.5±1.2	7.2±0.6	8.4±0.6	7.8±0.4
<u>Threonine</u>									
AVCN granular	8.7±0.9	12.3	11.1±1.4	10.7±1.1	10.6±1.1	10.8±0.3	10.3±0.7	9.6±0.6	11.3±0.7
AVCN subgranular	7.6±0.9		9.9	8.4±0.7	8.0±0.5	8.6±0.5	7.1±0.1	9.3±0.8	7.9±0.4
AVCN main	6.2±0.4	9.2±0.3 ^{dy}	7.2±0.3	7.4±0.3	6.4±0.3	7.7±0.3 ^{ex}	6.3±0.3	8.0±0.4 ^c	6.5±0.3
IN (ANR)	3.2±0.3	6.2	4.1±0.1	5.8±0.4 ^{dy}	3.8±0.3	6.1±0.8	3.2±0.3	7.0±0.4 ^{dy}	2.9±0.2
PVCN caudal	6.5±0.4	6.0±0.4	5.1±0.5	7.6±0.4	6.7±0.2	7.8±0.9	6.6±0.3	8.7±0.7 ^x	6.1±0.4
DCN deep	5.7±0.5	8.0±0.3 ^{ex}	6.5±0.4	6.7±0.2	6.4±0.2	7.2±0.2 ^x	6.1±0.3	8.0±0.2 ^{ex}	6.6±0.4
DCN fusiform soma	7.3±0.7	9.4±0.7	8.6±0.4	7.4±0.3	8.1±0.4	8.2±0.3	8.1±0.3	9.3±0.4	8.9±0.3
DCN molecular	8.3±0.8	11.4±0.7 ^c	11.0±0.7	9.1±0.2	10.6±0.7	9.0±0.4	9.3±0.3	10.6±0.5	10.4±0.3
<u>Arginine</u>									
AVCN granular	1.8±0.1	1.8	2.1±0.02	2.4±0.3	2.2±0.2	2.3±0.5	1.9±0.2	2.1±0.2	2.3±0.2
AVCN subgranular	1.7±0.1		2.0	2.1±0.1	1.8±0.1	2.0±0.2	1.6±0.2	1.7±0.04	1.6±0.1
AVCN main	1.5±0.1	1.7±0.1 ^x	1.3±0.02	1.8±0.1 ^x	1.5±0.1	1.8±0.1	1.4±0.1	1.7±0.1 ^x	1.4±0.1
IN (ANR)	1.2±0.1	2.0	1.2±0.1	2.4±0.3 ^c	2.0±0.4	1.7±0.1 ^{ex}	1.3±0.1	2.0±0.1 ^{dy}	1.2±0.1
PVCN caudal	2.3±0.1	2.3±0.2 ^x	1.6±0.1 ^d	2.7±0.2	2.2±0.1	2.8±0.5	2.0±0.1	2.7±0.1 ^x	2.0±0.1
DCN deep	1.3±0.1	2.0±0.2 ^c	1.5±0.1	1.7±0.1	1.5±0.1	1.6±0.2	1.4±0.1	1.5±0.1	1.3±0.1
DCN fusiform soma	1.5±0.1	2.0±0.2	1.7±0.1	1.6±0.1	1.5±0.1	1.6±0.2	1.4±0.2	1.5±0.1	1.6±0.1
DCN molecular	1.8±0.1	2.5±0.5	2.1±0.1	2.0±0.1	2.1±0.1	1.8±0.3	1.6±0.1	1.9±0.1	1.8±0.1

^a Details as in Table 1. Numbers of samples providing data were the same for taurine as for GABA, for glutamine as for glutamate, and for serine, threonine and arginine as for aspartate and glycine.