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Amygdala connections with jaw, tongue and laryngo-pharyngeal premotor neurons

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Abstract

As the central nucleus (CE) is the only amygdaloid nucleus to send axons to the pons and medulla, it is thought to be involved in the expression of conditioned responses by accessing hindbrain circuitry generating stereotypic responses to aversive stimuli. Responses to aversive oral stimuli include gaping and tongue protrusion generated by central pattern generators and other premotor neurons in the ponto-medullary reticular formation. We investigated central nucleus connections with the reticular formation by identifying premotor reticular formation neurons through the retrograde trans-synaptic transport of pseudorabies virus (PRV) inoculated into masseter, genioglossus, thyroarytenoid or inferior constrictor muscles in combination with anterograde labeling of CE axons with biotinylated dextran amine. Three dimensional mapping of PRV infected premotor neurons revealed specific clusters of these neurons associated with different oro-laryngo-pharyngeal muscles, particularly in the parvicellular reticular formation. CE axon terminals were concentrated in certain parvicellular clusters but overall putative contacts were identified with premotor neurons associated with all four oro-laryngo-pharyngeal muscles investigated. We also mapped the retrograde trans-synaptic spread of PRV through the various nuclei of the amygdaloid complex. Medial CE was the first amygdala structure infected (4 days post-inoculation) with trans-synaptic spread to the lateral CE and the caudomedial parvicellular basolateral nucleus by day 5 post-inoculation. Infected neurons were only very rarely found in the lateral capsular CE and the lateral nucleus and then at only the latest time points. The data demonstrate that the CE is directly connected with clusters of reticular premotor neurons that may represent complex pattern generators and/or switching elements for the generation of stereotypic oral and laryngo-pharyngeal movements during aversive oral stimulation. Serial connections through the amygdaloid complex linked with the oro-laryngo-pharyngeal musculature appear quite distinct from those believed to sub-serve fear responses, suggesting there are distinct ‘channels’ for the acquisition and expression of particular conditioned behaviors.

Keywords

amygdala; reticular formation; taste; pseudorabies; larynx

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The central nucleus of the amygdala (CE) is the caudolateral component of a large striatal-like forebrain region, the extended amygdala (Alheid and Heimer, 1988), which lies behind and beneath the ventral striatum and pallidum. The CE has long been recognized as a critical structure in the expression of conditioned responses linked to associative learning processes thought to be performed by the basolateral nuclear complex of the amygdala (Ehrlich et al., 2009, Pape and Pare, 2010). More recent studies implicate the CE in the actual learning processes, particularly in fear conditioning (Wilensky et al., 2006, Zimmerman et al., 2007). The main components of conditioned fear responses – potentiated startle, freezing, alterations in autonomic function – are widely thought to be mediated by direct CE projections to the nucleus pontis caudalis, substantia nigra, and dorsal medulla respectively, as well as indirectly via the ventral periaqueductal gray (Sah et al., 2003).

The CE sends substantial projections to the pontine and medullary parvicellular reticular formation ventral to the nucleus tractus solitarius (Krettek and Price, 1978, Thompson and Cassell, 1989, Dong and Swanson, 2003, Yasui et al., 2004) though little is known about the organization of these connections. Combined anterograde and retrograde axon tracing studies (Takeuchi et al., 1988a, Takeuchi et al., 1988b, Yasui et al., 2004) have suggested that CE axons contact trigeminal pre-motor neurons in the parvicellular reticular formation but there is no direct evidence linking the CE to specific jaw muscles. Viral tracing studies have also suggested indirect connections between the CE and the hypoglossal nucleus (Ugolini, 1995) and the nucleus ambiguus (Van Daele and Cassell, 2009) but no direct or indirect link has been established.

Connections between the CE and premotor neurons in the pontine and medullary reticular formation may subserve the role of the CE in taste aversion learning (Yamamoto, 2007). Responses to ingested unpalatable tastes are largely stereotypic orofacial movements such as mouth gaping and complex tongue movements (Grill and Norgren, 1978, Travers and Norgren, 1986, Dinardo and Travers, 1994). Studies using electrophysiology, viral tract-tracing, and Fos expression have identified in the pontomedullary reticular formation a complex network of pre-motor and higher-order inhibitory neurons involved in generating and switching between stereotypic ingestive and rejection movements of the jaw and tongue (DiNardo and Travers, 1997, Travers et al., 2000, Chen and Travers, 2003). Masticatory and tongue movements are also closely linked with pharyngeal and laryngeal muscles involved in swallowing with neurons located in the dorsal intermediate reticular formation (IRT) being active, for example, during swallowing and licking (Travers et al., 2000) and sending axons to multiple motor nuclei (e.g. Li et al., 1993). CE input to central pattern generators in the PCRT and/or IRT cells connected with several cranial nerve motor nuclei may provide the substrate by which the amygdala can co-opt stereotypic rejection movements during taste aversion learning.

The CEA, and the amygdala as a whole, plays a central role in innate fear responses, and the acquisition and expression of conditioned aversion (Davis, 1997, Maren, 2001) involving a wide variety of modalities. Recently, taste aversion has been included in this model with lesions of the amygdala disrupting taste aversion through attenuation of neophobia (Reilly and Bornova, 2005). However, responses to novel and aversive oral stimuli are different than those seen as fear responses such as freezing, potentiated startle, and changes in autonomic function (Davis et al., 1994). How the amygdala as a whole and the CE in particular differentially generates these responses is not known. One possibility is that intrinsic circuits provide separate, independent subsystems for different modalities of stimuli and different response clusters.

The present study sought to determine two features of the reticular connections of the central nucleus: i) whether the CE innervates premotor pattern generators in the pons and medulla

that could mediate complex ingestive/rejective behaviors involving lingual, masticatory and laryngopharyngeal muscles ; and ii) whether these connections are part of a distinct system through the lateral and medial CE that is independent of the circuit involving the lateral nucleus and the capsular or shell portion of the CE. Since there are no clear-cut anatomical criteria for identifying pattern generators in the pons and medulla, we first mapped in three dimensions the initial retrograde trans-synaptic infection of the reticular formation by pseudorabies virus (PRV) following inoculation of the genioglossus, masseter, inferior pharyngeal constrictor, and thyroarytenoid muscles to identify specific clusters of reticular neurons premotor to motoneurons innervating these muscles. This was followed by a combination of anterograde axonal tracing from the central nucleus with biotinylated dextran-amine (BDA) and transneuronal viral labeling from the oro-laryngo-pharyngeal muscles. Additionally, we have mapped the pattern of progressive trans-synaptic infection of cells in the principal nuclei of the amygdala.

EXPERIMENTAL PROCEDURES

Investigations were carried out on 88 male, Sprague-Dawley rats (Harlan), 290 to 320 gms weight. All surgical procedures were approved by the University of Iowa ACUC and conducted under ketamine/xylazine anesthesia (80mg/kg intraperitoneal) using aseptic surgical techniques. Post-operative care and monitoring followed NIH guidelines. Animals were euthanized with an overdose of Nembutal (100mg/kg sodium) followed by exsanguination.

Virus injections

Unilateral injections of the attenuated Bartha strain of pseudorabies virus (PRV) labeled with green fluorescent protein (GFP, kindly provided by Dr. Lyn Enquist, Princeton, NJ, USA) were made as a single bolus of 6×10^{23} pfus directly into the posterior cricoarytenoid (PCA), the thyroarytenoid (TA), genioglossus (GG), masseter (MS) and inferior constrictor (IC) muscles, as described previously (Van Daele and Cassell, 2009). For PCA injections, the larynx was exposed by a midline skin incision, retracted and slightly rotated to expose the lateral aspect of the cricoid cartilage, and the needle of a beveled Hamilton microsyringe inserted into the PCA to a depth of 2 mm. For masseter injections, the syringe needle was passed transcutaneously until contact was made with the mandible. The needle was then withdrawn about 2mm and the intramuscular injection was made. Genioglossus injections were made by inserting the needle of a Hamilton syringe through the underside of the tongue roughly parallel to and about 1mm lateral to the midline. A bolus of virus was injected at roughly the midpoint of the organ. Pharynx injections were made after a midline skin incision had exposed the visceral neck compartment. The trachea was gently pulled forward and injections made under direct vision into the inferior constrictor muscle. Most PRV injected animals were sympathectomized bilaterally at the time of injection though over half the GG and MS cases were sympathectomized only on the injected side. As PRV infection patterns were identical for unilaterally and bilaterally sympathectomized animals, and our previous studies (Van daele and Cassell, 2009) showed that sympathectomy did not affect amygdala labelling, the former was preferred as it reduced the number of surgical procedures animals were subjected to. Sympathectomy was achieved by avulsion of the superior cervical sympathetic ganglia: successful sympathectomy was confirmed by the presence of ptosis.

Groups of animals were euthanized at 3, 4 and 5 days after PRV injection by intraperitoneal injection of pentobarbital (100mg/kg). Following perfusion fixation with 4% paraformaldehyde and 0.1% glutaraldehyde, frozen sections of the brain 50 microns thick were cut in the horizontal or coronal plane. Viral infection of neurons was detected through immunocytochemical labelling of the green fluorescent protein. Brain sections were

incubated in a biotinylated goat anti-GFP antibody (Vector Laboratories) diluted at 1:1000 for 24 hours at 4° C. Final staining was done using avidin-HRP reagents (Vector Elite ABC kits) and 0.01% 3,3'-diaminobenzidine. Sections were washed and mounted in serial order on slides, dried, dehydrated and cleared and permanently mounted in Permount. Alternate or selected slides were counterstained with cresyl violet.

A total of 22 PRV injected animals had received prior microiontophoretic injections of biotinylated dextran amine (BDA- Chemicon as a 10% aqueous solution of 10kD or a 50:50 mixture of 3kD and 10kD molecular forms) into the central nucleus of the amygdala (CE). Injections of BDA were delivered into the medial subdivision (CEM) of the central nucleus through stereotactically placed glass micropipettes with external tip diameters in the range of 3–10µm. A continuous 1–3µA current was passed via a tapered silver wire fitted into the pipette for 15 seconds to 2 minutes after which the pipettes were withdrawn in 25µM increments every 10 seconds. This procedure resulted in small, spherical deposits of BDA of between 10 to 50µm in diameter, depending on current strength. Animals were allowed to survive for 10 to 12 days before being given PRV injections as described.

BDA was detected by incubating sections for 1–2 hours in Vectastain ABC reagent in PBS containing between 0.1 and 0.5% Triton-X depending on section thickness. After multiple washings in PBS, sections were incubated in 0.1% 3,3' diaminobenzidine (Sigma) in 0.05M Tris buffer containing 0.5% cobalt chloride and nickel ammonium sulphate for 20–30 minutes. Reaction was completed by adding 0.03% hydrogen peroxide at 40µl per 10ml of solution. Following this most sections were washed, dehydrated and mounted in Permount.

Ibotenic Acid injections

Eight animals were given two unilateral injections of ibotenic acid into the dorsal or ventral medulla (four animals each) prior to inoculation of PRV into the masseter or tongue (4 animals each). These animals were not sympathectomized. Between 0.1 and 0.2 µL of Ibotenic acid (0.03M in distilled water) was injected through a Hamilton syringe at approximately 2 to 2.5 mm from the midline in two locations; one just caudal to the descending limb of the facial nerve and the other at approximately the level of the obex. Animals were allowed to recover for 5–7 days before receiving inoculations of PRV into the tongue or masseter on the same side as the ibotenic acid injection. Two additional animals were given injections of 0.9% saline into the PCRT followed by PRV inoculations of the tongue. Animals were euthanized 5 days after viral inoculation and brain tissue processed as described above. Serial 50 micron thick horizontal sections were immunostained for GFP; alternate sections were counterstained with cresyl violet.

Sections were examined with a Nikon Labophot microscope and for each case, every third or fourth section was examined under 10X magnification and the distribution of labeled neurons and the outlines of major brain structures plotted digitally. Identification of structures was done on the basis of staining characteristics and thorough comparison with a large set of reference sections and atlases. All digital photomicrographs were taken with a Pixera Pro150ES camera and digitally processed using Adobe Photoshop version 7.0. No artificial enhancements were made during processing though several pictures were digitally retouched to remove dust spots and other blemishes: none of this affected image data.

Cell counts

The degree and progression of PRV infection of the amygdala was determined by counting all GFP-immunoreactive nucleated cell fragments greater than 10 micron in diameter in a sample of sections. The 10 micron cut-off was used to eliminate counting of GFP-immunoreactive glia. For the CE and the basolateral nucleus, a single horizontal section was

used, selected by determining the point where the rostral border of the stria terminalis (in cross-section) was aligned with the rostral tip of the optic tract (in cross-section). This section corresponded to the approximate dorsal-ventral midpoint of the CE and included a large extent of the medial, lateral (CEL) and capsular (CELC) divisions. In sections sampled on this basis, a substantial portion of the magnocellular (BLM) and parvocellular (BLP) divisions of the basolateral nucleus was also present and counts were made in these structures. For the lateral nucleus (LN), GFP-immunoreactive cell fragments greater than 10 micron in diameter were counted on alternate horizontal sections commencing with the upper edge of the CE and ending with the disappearance of the LN when the longitudinal association bundle merges with the external capsule.

Reconstruction and analysis

The location and three-dimensional distribution of PRV infected neurons in the pons and medulla were determined in one of the animals receiving PRV inoculations into the pharynx, larynx, masseter or tongue. The particular case was selected as showing the typical pattern (late 4 day) of brainstem labeling and the presence of small numbers of infected cells in the medial central nucleus. For each case, the distribution of infected neurons in the reticular formation (small dots in Figs 10 and 11.) and infected motoneurons (large dots in Figs. 10 and 11) was plotted from serial 50 micron thick horizontal sections commencing at the ventral border of the NTS and ending at the ventral border of the facial nerve nucleus. The cross-sectional profile of the facial nerve was also drawn from each section. Plots from each section were stacked in Image J (version 1.410, NIH) and reconstructed as a three-dimensional image using Metamorph© software (version 7.63)

The distribution and arborizations of CE axons in the pons and medulla were reconstructed in nine BDA cases in their entirety from serial 150µm thick horizontal sections at high power magnifications. All axon segments in individual sections were either traced onto paper using a camera lucida attachment (final magnification 23X) or photographed through a 10X /0.2 N.A. lens using a Pixera 400L digital camera attached to a Nikon Labophot microscope. Accuracy and completeness of drawing was checked using 20X–100X objectives and various landmarks, e.g. midline, ventricular and surface edges, large blood vessels, etc., were additionally marked. Nuclear and other structural boundaries were marked on a portion of sections that were lightly counterstained with cresyl violet. Otherwise, on uncounterstained sections, white matter structures were located and marked under cross-polarized illumination. Paper tracings were digitized full-size by scanning with a Hewlett Packard Scanjet 5400C and saved as individual files. High-resolution reconstruction was done on a regional basis by merging groups of tracing files using Adobe Photoshop 6.0 or a customized version of the 3-D program VoxBlast. Overall reconstruction was done by pasting regional reconstructions together using Image J and Metamorph. All reconstructions were repeatedly cross-checked with original material for fidelity and continuity. All axons reported in this study had to be traceable back to a cell body in the medial central nucleus for inclusion. In a number of instances, short (5–20µm) segments of axons could not be identified and broke the continuity of reconstructed branches. These were located mostly on the surfaces of sections and were presumably lost during sectioning: they are marked by dotted lines in the figures. In a number of cases where more than one neuron was filled with BDA, all axons segments were drawn and reconstructed in total, individual axon arborizations being identified by color-coding.

In the double-stained material sectioned at 50µm, short axon segments and immunostained perikarya and dendrites were drawn in various regions of the medulla with 40X (final magnification 525X) or 63X (final magnification 805X) objectives on adjacent pairs of sections. In most cases, axons (only) were followed, drawn, and reconstructed from 3–5 sections either side of the original pair. Putative appositions between axons and

immunolabeled elements were confirmed by focusing through sections with either 63X dry or 100X oil immersion objectives.

RESULTS

Brainstem PRV infection following larynx, pharynx, tongue and masseter injections

All animals sacrificed at 3 days p.i. had labeling of motoneurons in the respective cranial nerve motor nucleus (FIG.1). In cases with PRV inoculation into the MS, infected neurons were initially (3 days) found only in the ipsilateral ventrolateral trigeminal motor nucleus (moV) but by 4 days this had expanded to include almost the whole lateral half of the moV (Fig.1A; see also (Giaconi et al., 2006). A small number of infected cells were seen in both the facial (NVII) and hypoglossal (NXII) motor nuclei by 5 days p.i. Following tongue injections, which presumably inoculated PRV into both the intrinsic muscles and genioglossus, a considerable number of infected neurons was present in NXII at 3 days p.i.; by 5 days p.i., PRV-infected neurons dominated the caudal half of NXII (Fig.1B). Unlike other inoculations, only a few infected motoneurons were seen outside the NXII. These were located (in order of frequency) the nucleus ambiguus (NA), NVII and moV.

Following IC injections, infected neurons in 3 day animals were mostly confined to the semi-compact division of the NA (NAc, Fig.1C), consistent with retrograde tracing studies (Bieger and Hopkins, 1987; Bao et al., 1995), though infected cells were also seen in the loose division at this time point and later. In animals sacrificed at five days p.i., infected neurons were present in the compact NA, and a few cells were consistently present in the hypoglossal nucleus. In the TA and PCA inoculated cases, infected neurons were restricted to the loose division of the nucleus ambiguus (NAI) though by 4 days, additional infection was seen in the dorsomedial part of the compact division (Fig.1D; see also Van Daele and Cassell, 2009) and in several of the cases sacrificed at 5 days p.i., small numbers of infected cells were present in the hypoglossal and facial nuclei. In all TA, PCA and pharynx injected cases, infection of the NA contralateral to the injected side was present.

Infection of neurons in the parvicellular, gigantocellular and intermediate reticular formation, the nucleus tractus solitarii (NTS), the Botzinger complex, the A7/subceruleus region and the locus ceruleus was observed in almost every case at 4 days p.i. as described elsewhere (e.g. Ugolini, 1995; Fay and Norgren, 1997a,b,c; Giaconi et al., 2006; Van Daele and Cassell, 2009). In the MS and GG injected cases, infected neurons were also consistently present in the spinal and mesencephalic trigeminal nuclei, and in all 4 day p.i. cases involving larynx, pharynx and tongue injections infected cells were identified in the medial and ventrolateral preoptic regions and the ipsilateral parvicellular portion of the paraventricular hypothalamic nucleus.

Infection of the amygdala after oro-pharyngeal muscle injections of PRV

Irrespective of the site of inoculation, PRV infected cells were first seen in the amygdala in animals sacrificed at 4 days (96–108 hours post inoculation) (FIGS2A and B, 3A and B). Initial infection was almost exclusively confined to the ipsilateral CEM though occasional PRV infected cells were seen in CEL, the parvicellular basolateral nucleus (BLP) and the amygdalo-piriform transition area (APir; FIG4A; Table 1). Some quantitative differences in the numbers of CEM cells infected at 4 days were evident with pharynx injections infecting the fewest cells (FIG4A) and tongue injections the most. Some subtle differences in the distribution patterns of infected CEM neurons were observed depending on which muscle was inoculated but these were not systematically compared. In all cases, neurons were concentrated in the rostral two-thirds of CEM (FIG2 and 3).

In animals sacrificed at 5 days (120+ hours), considerable expansion of both the numbers and distribution of infected cells had occurred (FIGS2C and D; 3C and D; 4B). In the APir, BLP and CEM there was a dramatic increase in the number of infected cells and infection had now spread to include CEL in every case (Table 1). As with CEM, there was considerable variability in the numbers of infected neurons in CEL, with the tongue-injected cases consistently having the greatest number of infected cells (Table 1). In all cases, infected neurons were identified in the other portions of the extended amygdala, including the substantia innominata (SI) and posterolateral bed nucleus of the stria terminalis (BNSTpl), but not the interstitial nucleus of the posterior limb of the anterior commissure (IPAC; FIG4A). In contrast to the infection pattern seen in CEM, the distribution of infected cells in CEL differed between inoculation sites: tongue inoculations resulted in infected neurons concentrated medially (FIG2B) while masseter inoculations produced infection in cells located rostrally (FIG2D); TA inoculations gave infected cells concentrated caudolaterally (FIG3B) whereas IC inoculations resulted in infected cells concentrated rostromedially (FIG3D). While occasional infected cells were seen in CELC in two-thirds of cases, they rarely exceeded one cell per section and a third of cases had no infected cells in CELC. Only very rarely were infected cells seen in the lateral nucleus (LN) even though every section containing this nucleus was examined. The greatest number of infected LN cells was found in the tongue cases where the heaviest infection of CEM, CEL and CELC was present. In one heavily infected tongue case, a substantial number of infected neurons were identified in the magnocellular basolateral nucleus (BLM; Table 1).

Effects of ibotenic acid lesions of the PCRT on amygdala infection

Previous studies (Yasui et al., 2004) indicated that the PCRT was a site of interaction between central nucleus efferents and trigeminal premotor neurons and was thus targeted by ibotenic acid (IBO) injections. In all cases, IBO injections resulted in roughly spherical lesions in the PCRT where few neuronal perikarya were identifiable and in most cases a readily discernible gliosis was present (FIG5). Infected neurons were present in the moV and NXII in the respective cases, indicating infection of primary motor neurons had occurred. The IBO lesions generally extended from the portion of the PCRT caudal to the facial nerve to the level of the obex, to include almost the full extent of the dorsal PCRT. PRV-infected neurons were very sparsely distributed in the PCRT and fewer were found in the NTS and hypothalamus compared to the numbers usually found at 5 days post inoculation. However, in all but one case, there was a considerable attenuation of infection of the forebrain ipsilateral to the IBO lesions.

Two of the four cases with tongue inoculation (Table 2; Tongue IBO, FIG 5), had extensive gliosis of the dorsal PCRT, and had very reduced numbers in the ipsilateral CE and no contralateral infection: this contrasted with the usual pattern of CE infection at 5 days p.i. (Table 1). One case, with a smaller, irregular lesion of the rostral dorsal PCRT, and including part of the caudal NTS, had a pattern of amygdala infection consistent with the usual 5 day pattern. Significantly, the lesion in this case did not involve much of the PCRT adjacent to the facial nerve, unlike the other three cases.

In all four of the animals with PRV inoculations into the MS muscle, the IBO lesions were located in the caudal two-thirds of the dorsal PCRT (FIG5). In this area, which was always heavily infected following MS inoculations, few if any infected neurons were detected (FIG5). In all but one of these cases, the numbers of infected CEM neurons was less than 30% of the lowest number seen at 5 days p.i. (5 day case 3, Table 1) while the number of infected CEL neurons was significantly reduced.

The control injections of saline vehicle (e.g. Tongue Control, FIG5) did not appear to affect the pattern of infection in the forebrain and hypothalamus.

Medial central nucleus (CEM) projections to the pontomedullary reticular formation

The attenuating effect of IBO lesions of the PCRT suggested this may be principle site from where PRV was taken up by CEM neurons after inoculation into the oro-pharyngeal musculature. To determine how widespread CEM innervation of the pontomedullary reticular formation was and whether CEM axons showed preferential contact with specific cell groups, small iontophoretic injections of BDA restricted to CEM were made (FIG 6). Because of the passage of cortico-medullary axons close to and through the CE, the three cases examined all had prior ipsilateral lesions of the insular cortex from roughly bregma to 3mm caudal to bregma to eliminate *en passant* labeling of the axons by CE injections.

In all three cases, BDA injections labeled a small cluster of neurons (between 20 and 165 cells) in CEM (FIG 6); the pattern of axonal and terminal labeling in the pons and medulla was virtually identical in each case. The overall distribution of central nucleus axons in the horizontal plane of the pons and medulla at 7 different dorso-ventral levels is shown in FIG7. The overall distribution of axons and axon terminals in the pons and medulla was broadly consistent with previous anterograde studies (Krettek and Price, 1978, Gray and Magnuson, 1987, Cassell and Gray, 1989, Danielsen et al., 1989, Koch and Ebert, 1993, Batten et al., 2002, Geerling and Loewy, 2006) with labeled elements (predominantly axons) in the subrostral, medial (mNTS), and commissural (comNTS) subnuclei of the nucleus tractus solitarii, the parvicellular and intermediate reticular zones, the caudo-ventral and dorsal portions of the mediodorsal reticular area (MDd), and the rostroventrolateral medullary region. Except for an occasional few, fine fibers, no labeled elements were identified in the cellular regions of the motor trigeminal nucleus, the hypoglossal nucleus (NXII; FIG.7 D,E) the dorsal motor nucleus of the vagus (NX; FIG. 7B–D), the facial nucleus (NVII; FIG6 G) or the nucleus ambiguus (NA; FIG.7G). Consistent with previous studies (Koch and Ebert, 1993), no labeled elements were observed in the pontine nucleus caudalis (FIG. 7F, G) though a few fibers were consistently seen in the nucleus reticularis gigantocellularis.

Three-dimensional reconstruction (FIG.8) and high power examination of CE axons in the reticular formation (FIG. 6B–D; FIG8) revealed substantial differences in the density and distribution of branches ending in putative *bouton terminaux*. Axon and terminal labeling in the PCRT was present at all dorsoventral levels, from the zone ventrolateral to the NTS to the dorsolateral aspects of the nucleus ambiguus. Axon segments in the PCRT ran rostro-caudally with putative *boutons en passant* as well as small side branches (FIG. 6C) but in three regions there was an obvious concentration of side branches ending in spray-like endings (FIG. 6D) with numerous short axon branches with *bouton terminaux*. The most rostral of these concentrations was located just caudal to the descending root of the facial nerve in a region commonly referred to as PCRT alpha (FIG. 7E and zone 1 FIG. 8). This terminal zone was roughly 1mm in diameter and extended ventrally to the perifacial zone surrounding the facial nucleus (FIG. 7G). The center of the second zone (zone 2 FIG. 8) of concentrated branches and boutons was located at roughly midway between the descending root of the facial nerve and the obex. This concentration was on a slightly higher horizontal plane than the rostral concentration but was of a similar size. The third concentration was centered at approximately 1mm behind the obex and lateral and ventral to the hypoglossal nucleus (zone 3, FIG. 8). In between the zones 2 and 3, axon segments possessed few side branches. The most caudal concentration (zone 4 FIG. 8) of boutons was more ventrally located than zone 3 and smaller in size.

At the caudal end of the PCRT zone 4 terminal field, most of the axons passing through the PCRT made a sharp, almost right-angled, bend medially and ascended through MDd towards the midline where a second bend turned them rostrally (FIG.7C;FIG.8A). Here the axons traveled for up to 4 mm alongside the central canal and caudal one-half of the fourth

ventricle (FIG. 7A;FIG.8). Numerous short branches with *bouton terminaux* could be identified in the A2/C2 region (Wallace et al., 1992) but only occasionally in the mNTS.

In the intermediate reticular formation (IRT), most axonal labeling was of long segments with very few side branches and occasional apparent *boutons en passant* (FIG.7C and D; FIG. 8A) However, side branches were more frequent in the areas of the IRT adjacent to the concentrations of terminal labeling in the PCRT. In addition to these concentrations, smaller clusters of side-branches were evident though in contrast to the larger concentrations, these were not consistently found in every case.

Reconstruction of CEM axons in the pontomedullary reticular formation

To determine whether CEM inputs to the pontine and medullary reticular formation are organized in a uniform, parallel fashion or as specific axon branches, the ponto-medullary segments of nine axons derived from BDA-filled CEM neurons (FIG. 9A) were reconstructed from serial 150µm thick sections (FIG. 9B–E). All nine had a similar trajectory and pattern of branching though all differed in the extent to which the NTS was innervated. In all cases, CEM axons gave rise to between 2 and 4 parallel branches as they passed caudal to the descending root of the facial nerve (FIG. 9B and E). In all cases, one or two branches coursed through the dorsal portion of the IRT, giving rise to regular side branches that ended in localized spray-type endings (arrows FIG. 9 B and E). Other long axon branches passed more ventrally through the PCRT and some differences were observed in their branching patterns. Comparing the axons shown in Figures 9B, C and E, one axon (E) had extensive branches in the PCRT alpha (zone 1) while the other (B) had none. This latter axon segment continued throughout the length of the PCRT with only occasional side branches. Additionally, no branches were traced to the NTS though a series of long branches were identified in MDd. The axon shown in Figure 9B, on the other hand, gave rise to small side branches in the PCRT just dorsal to the NA (open arrow in FIG. 9E), passed through MDd then coursed rostrally through the ventral and medial NTS (FIG. 9D).

Pre-motor neurons in the pons and medulla

The concentration of axon branches and terminals from CE neurons in patches in the pons and medulla (FIG. 8) suggested potential selective innervation of groups of pre-motor neurons there rather than a uniform pattern of innervation. Three-dimensional plotting of the distribution of PRV infected premotor neurons at 96 hours post-inoculation revealed highly distinctive patterns of distribution for each inoculation site and suggested premotor neurons were clustered or banded in the pons and medulla.

In the horizontal plane (FIG.10A–D), it was evident that the relative proportions and distribution of neurons in the PCRT and IRT differed along their rostrocaudal axes between inoculation sites. TA, PCA and MS inoculations resulted in concentrations of infected neurons in PCRT while tongue and IC inoculations gave a more evenly distributed pattern of infection across both PCRT and IRT. However, clear clustering of populations of premotor neurons was evident in the rostral-caudal and dorsal-ventral planes (FIGS. 10 and 11A–D) and rostral to the obex, the populations of neurons in both PCRT and IRT infected from different muscle injections were largely non-overlapping. In the IRT, infected cells formed small clusters some 250–500µm in diameter whereas in PCRT the clusters were roughly a millimeter in diameter (FIGS. 10 and 11).

PCRT neurons infected from laryngeal and IC inoculations were mostly concentrated caudal to the obex and in the dorsal and ventral parts of the rostral PCRT. Apart from a few scattered cells, there were few infected cells in the first two millimeters caudal to the descending limb of the facial nerve. In contrast, PCRT neurons infected by tongue and MS

inoculations were concentrated in the central portion of the rostral PCRT and appeared to have little overlap with laryngeal and pharyngeal premotor neurons. Furthermore, tongue muscle inoculations resulted in infected cells concentrated in a cluster in PCRT just caudal to the descending limb of the facial nerve while neurons infected by MS inoculations were concentrated just caudal to this in a non-overlapping cluster (FIGS. 10 C and D; 11 C and D). Infected neurons from tongue inoculations were relatively sparse caudal to the obex except for a loose cluster that extended across both PCRT and IRT at the level of the obex. MS inoculations, however, produced a dense concentration of PRV infected cells in the PCRT extending for 2mm caudal to the obex (FIGS. 10D and 11D).

In the IRT, PRV infected cells were concentrated caudal to the obex following TA inoculations (FIG. 10A). Contrasting with this, IC inoculations infected IRT cells *rostral* to the obex with a distinct clustering of cells rostrally (FIG. 10B). Tongue and MS (FIG. 10C and D) inoculations both resulted in a concentration of infected cells in IRT at the level of the obex with tongue inoculations labeling neurons more neurons rostrally. In the most rostral regions of the IRT, distinct clusters of infected neurons were present in the dorsal region just beneath the NTS (FIGS. 10C and D; 11C and D).

CE projections to pre-motor neurons in the pons and medulla

Independently, the distribution patterns of CE axons in the pons and medulla and the clusters of premotor neurons appeared to predict specific areas of overlap. For example, the zone 1 terminal field (FIG. 8) appeared to overlap with the rostral clusters found after tongue (FIG. 10C, 10C) and masseter (FIG. 10D) inoculations. To examine whether CEM axons preferentially innervated certain clusters of premotor neurons and not others, anterograde labeling of CEM axons with BDA (FIG. 12A–D) was combined with PRV inoculation into oro-laryngo-pharyngeal muscles.

Dark-field illumination enhanced visualization of the interactions between CEM axons and PRV infected cells and the clustering of PRV infected cells was much more evident (FIGS. 13A and E and 14A and E). Despite the presence of concentrations of terminals derived from CEM axons, the combined BDA/PRV experiments demonstrated that CEM axons make putative synaptic connections with premotor neurons in both PCRT and IRT related to laryngeal, pharyngeal, tongue and jaw muscles. In the PCRT (FIGS. 13A–D), the concentration of axon terminals in zone 1 (just caudal to the facial nerve) produced numerous putative contacts with the dominant cluster of premotor neurons infected by tongue inoculations (FIG. 13B–D) as would be predicted but axon terminals here also contacted premotor neurons infected by TA and IC inoculations (FIG. 14B and F). Similarly, numerous contacts were evident in the zone 2 terminal field with the dominant cluster of neurons infected by MS inoculations (FIG. 13E and F) but contacts were also seen with neurons infected following tongue (FIG. 13A and C) and pharynx (FIG. 14A and C). The more caudal concentrations of CEM axon terminals (zones 3 and 4) made contacts with infected neurons from all inoculation sites (FIG. 13D and H; FIG. 14D).

In the IRT, CE axons gave rise to branches with terminal arborizations that interacted most commonly with clusters of small diameter neurons (FIG. 15 A–C). Contacts visible at the light microscopic level were mostly axodendritic contacts. Again, these putative contacts were identified after TA, IC, tongue and MS inoculations of PRV.

DISCUSSION

The major findings from the present studies are: i) retrograde transynaptic tracing with pseudorabies virus from select oro-laryngo-pharyngeal muscles reveals a multi-synaptic connection from the amygdala to the ponto-medullary reticular formation through the central

amygdaloid nucleus that does not include the lateral or magnocellular basolateral amygdaloid nuclei; ii) medial central nucleus projections to the ponto-medullary reticular formation innervate clusters of premotor neurons in the IRT that may represent coordinating units for oral and laryngo-pharyngeal activity (Travers et al., 2005) and in the PCRT that may represent central pattern generators for stereotypic oral and laryngo-pharyngeal movements in response to aversive oral stimuli; iii) while individual CEM axons may selectively innervate certain clusters of premotor neurons, the overall trend is for a parallel innervation of premotor clusters in both the IRT and PCRT. This multi-synaptic circuit may represent the pathway by which the amygdala accesses aversive response circuitry in conditioned taste aversion. Further, this circuit appears to be quite distinct from the lateral nucleus-lateral capsular CE pathway subserving responses to conditioned fear. In contrast to the view that the CE is a generic mediator of amygdala output, the CE thus may possess distinct channels through which responses specific to different conditioned behaviors are generated.

Transneuronal infection of the central nucleus

A key issue in interpreting the pattern of infected structures found after pseudorabies virus injections into peripheral targets, particularly after long survival times, is the level (second, third, etc) at which trans-synaptic infection loses much of the specificity conferred by the initially restricted inoculation. Infection of CEM was not seen earlier than 4 days (96 hours) p.i. and trans-synaptic infection of other amygdala nuclei no earlier than 120 hours p.i., raising the possibility that CEM neurons became infected through secondarily infected areas, notably the NTS. The patterns of initial motor neuron infection by PRV inoculated into the TA, masseter and tongue muscles found here were virtually identical to those reported in previous retrograde viral tracing studies (Bao et al., 1995, Dobbins and Feldman, 1995, Ugolini, 1995, Fay and Norgren, 1997a, b, Travers and Rinaman, 2002, Giaconini et al., 2006, Van Daele and Cassell, 2009). Likewise, the distribution of second- and third-order infected neurons in the reticular formation, at least in general terms, was consistent with those reports as well as with studies employing conventional retrograde tracing techniques from respective motor nuclei (e.g. Li et al., 1995; Dinardo and Travers, 1997; Travers et al., 2005). However, it is possible that the consistent infection of amygdala neurons following inoculation of PRV into the oro-laryngo-pharyngeal musculature reflects consistent infection of a core structure such as the NTS, lateral hypothalamus, etc or alternatively that pre-autonomic neurons are infected and it is these that lead to retrograde trans-synaptic infection of CEM.

The most compelling evidence suggesting that CEM infection following oro-laryngo-pharyngeal muscle PRV inoculation is largely due to trans-synaptic uptake from infected premotor neurons in the PCRT is that ibotenic acid lesions of the PCRT severely attenuated infection of CEM without a similar degree of attenuation of infection in the paraventricular and lateral hypothalamic nuclei (FIG. 5). Both these hypothalamic nuclei innervate the NTS and PCRT as well as pre-ganglionic neurons (ter Horst et al., 1984) and presumably were either trans-synaptically infected from the NTS or through other medullary structures, including those involved in sympatho-motor control (Kerman, 2008). Our previous studies (Van Daele and Cassell, 2009) showed that sympathectomy had little or no effect on forebrain labeling following TA inoculations of PRV, though hypothalamic infection was reduced. Consistent with this, a study tracing poly-synaptic circuits innervating the pancreas with PRV (Buijs et al., 2001) also reported that while vagotomy virtually eliminated infection of the CE, sympathectomy did not. This would suggest that PRV infection of second or third order neurons related to pre-ganglionic sympathetic neurons is not a significant source of infection for the CE but is for hypothalamic nuclei.

Studies using PRV retrograde trans-synaptic tracing of visceral innervation, for example, of the heart (Ter Horst et al., 1996), submandibular salivary gland (Jansen et al., 1992), pancreas (Buijs et al., 2001), liver (la Fleur et al., 2000), thyroid (Kalsbeek et al., 2000) and kidney (Zermann et al., 2005), in both non-sympathectomized and sympathectomized animals, show only modest infection of CE neurons after even long survival periods despite strong infection of the dorsal vagal motor nucleus, pre-ganglionic sympathetic neurons and the paraventricular and lateral hypothalamic nuclei. These studies all report varying degrees of infection of the PCRT. Heavy CE infection has been reported, however, following PRV inoculation into the stomach (Yang et al., 1999) though in these experiments, very heavy infection of the PCRT was noted (their Figure 2). Since complex oro-laryngo-pharyngeal functions like swallowing require concurrent changes in visceral function, reticular formation neurons provide a ready substrate for coordination of somatic and visceral activities (Altschuler, 2001, Lang, 2009). It appears very likely that CE infection following oro-pharyngeal muscle inoculation of PRV was not due to secondary infection of primarily autonomic, or autonomic-motor related structures, including the NTS, but through the direct innervation of premotor neurons. Further, CE infection after PRV inoculation of the viscera may be due in part to infection of PCRT neurons that coordinate somatic and visceral activity.

Distinct channels through the central nucleus

Division of the CE into two distinct zones based on modality processing has been proposed and is supported by connectional studies (Cassell et al., 1999). The dorsolateral portion of the CE, referred to as the lateral capsular division (CELC - (McDonald, 1982)) or 'shell' (Cassell et al., 1999) contains a number of intercalated cell clusters and is heavily innervated by the lateral amygdaloid nucleus (Pitkanen et al., 1995), auditory and visual cortical areas (Shi and Cassell, 1997) and perirhinal cortex (Shi and Cassell, 1999). Neurons in the shell send their axons to a narrow strip of pallidal like tissue sandwiched between ventral globus pallidus and CEM (Jolkkonen et al., 2001b) which in turn sends axons to the periaqueductal gray and the nucleus pontis caudalis (Koch and Ebert, 1993). In contrast, the lateral CE (or 'core') receives input from the gustatory portion of the dysgranular insular cortex (Shi and Cassell, 1997) and the caudolateral parvocellular basolateral amygdaloid nucleus (Savander et al., 1995), and sends projections to CEM (Jolkkonen et al., 1998) which innervates the diencephalon, midbrain, pons and medulla. The retrograde pattern of infection of PRV shown here is strongly consistent with a distinct separation of a lateral nucleus-shell-ventral globus pallidus intra-amygdaloid circuit and a posterior (parvocellular) basolateral – CEL – CEM circuit. In only a few instances were PRV neurons found in the lateral nucleus and the lateral capsular division of the CE after oro-laryngo-pharyngeal muscle inoculation, and this after greater than 5 day survival periods. In contrast, trans-synaptic infection of CEM – the first amygdala nucleus to be infected - at 4 days post-inoculation was invariably followed by CEL, APir and BLP infection. Axonal tracing studies have shown that the caudolateral part of BLP innervates both CEL and CEM though CEM contains mostly fibers passing into the stria terminalis (Savander et al., 1995). However, the time course of infection (Table 1) suggests that PRV was likely taken up by BLP projections ending on CEM neurons, as well as from CEL, as BLP infection was noted even with only modest infection of CEL. Likewise, the amygdalo-piriform transition area sends projections to both CEM and CEL ((Jolkkonen et al., 2001a)) and its early trans-synaptic infection probably arose from infected neurons in CEM.

The lack of retrograde spread of PRV to CELC (and the lateral nucleus) despite heavy infection of CEM and CEL calls into question the recent suggestion that the intercalated cell masses (ICM), which form the principal cell component of CELC, send axons to CEL and that the lateral nucleus innervates CEL, at least in the guinea pig (Pape and Pare, 2010). In

fact, axon tracing studies from the LN in rats (Pitkanen et al., 1995) and monkeys (Fudge and Tucker, 2009) show only a few axons in CEL and CEM while the major terminal field is in the lateral capsular division. Further, anterograde tracer injections into CELC label the ventral globus pallidus adjacent to CEM (Jolkkonen and Pitkanen, 1998, (Cassell et al., 1999) with few labeled axons extending into CEM itself. The cells of the ICM share numerous characteristics with CEL neurons, including being GABAergic (Sun and Cassell, 1993, Marowsky et al., 2005), possessing metabotropic glutamate receptors and calponin/caldesmon immunoreactivity (Agassandian and Cassell, 2008). While species variation in intra-amygdaloid connectivity may account for the lack of spread of PRV from CEM and CEL to CELC and the lateral nucleus in the rat, it is likely that CELC innervates the ventral globus pallidus, forming the same connectional relationship as CEL has with CEM. This would predict that PRV or other viruses that undergo retrograde trans-synaptic transport from brainstem sites to the ventral globus pallidus would lead to transneuronal infection of CELC and the lateral nucleus, but not CEL or CEM.

Segmental Organization of Premotor neurons

Two distinct premotor cell groups appear innervated by CEM: the dorsal intermediate reticular formation and the parvicellular reticular formation. The IRT has been proposed to be made up largely of premotor neurons with connections to multiple oral and laryngopharyngeal motor nuclei (Travers et al., 2000). These neurons may constitute a substrate for co-ordinating multi-component oro-laryngo-pharyngeal functions such as swallowing. PRV inoculations into the tongue, masseter, thyroarytenoid and inferior constrictor muscles all transynaptically infected neurons in the IRT. The use of horizontal sections revealed a clustering of infected neurons in the IRT with some topographical organization. Though double-labeling studies with different viral markers were not performed (though see (Van Daele and Cassell, 2009) the overlapping nature of infected cluster patterns (despite specific pattern trends for each muscle) from different inoculation sites is consistent with anatomical (Amri et al., 1990, Li et al., 1993) and physiological (Travers et al., 2000) evidence for IRT cells connecting with multiple motor nuclei. The inference here, however, is that *clusters* of IRT neurons may connect with multiple motor nuclei and each cluster may co-ordinate or switch activity between different oro-pharyngeal muscle groups. The methodology employed here could only resolve CEM innervation of clusters rather than of specific, muscle-related premotor neurons. This neuronal clustering does clearly match the branching pattern of CEM axons in the IRT which was based on side branches with spray-like terminal arborizations (FIG. 6D). However, the combined virus/ anterograde tracing data did not indicate specific innervation of any particular cluster. Instead, CEM innervation of IRT clusters appears to be general one, based on a parallel branching axonal system.

The grouping of PCRT neurons trans-synaptically infected from oro-laryngo-pharyngeal muscles was decidedly different from that seen in the IRT. Premotor neurons formed much larger clusters and these appeared to be segmentally organized along the rostro-caudal axis (FIGS.10 and 11). In addition, dorsoventral differences in the main groupings of infected neurons were observed and, particularly at pontine and rostral medullary levels, certain non-overlapping distinctions were evident. Segmentation of the hindbrain into rhombomeres occurs during development (Lumsden and Krumlauf, 1996) and it is possible that the clustering of premotor neurons is a relic of this embryonic compartmentalization. Mutations in transcription factor genes responsible for rhombomere development (e.g. *Krox 20*, *Hoxa2*) result in phenotypes that have deficits in respiratory and masticatory rhythms (Jacquin et al., 1996, Chatonnet et al., 2007) so it is possible that these larger groupings of neurons represent complex pattern generators for individual muscles or antagonistic muscle groups. Other anatomical and physiological evidence also supports these clusters

representing central pattern generators. For example, a group of neurons infected from masseter, but not other inoculation sites was consistently found situated dorsal to the facial nucleus and in the same plane as the motor trigeminal nucleus (FIG. 10). This group appears in the same location as a group of premotor neurons providing excitatory input to jaw-closing motor neurons (Li et al., 1993, Takamatsu et al., 2005). Similarly, TA or IC inoculations infected neurons in the PCRT adjacent to the NA but were rarely encountered in the more central region of the rostral medulla and pons where masseter and tongue related premotor neurons were concentrated. The region of PCRT dorsal to the NA is a site where swallowing can be initiated while this is not the case for central parts of the PCRT (Kessler and Jean, 1985). These observations would seem to support a suggestion that, at least in the pons and rostral medulla, pattern generators for specific muscles or antagonistic muscles are segregated from each other in the PCRT.

As in the IRT, no evidence was found in the organization of CEM axons and terminals in the PCRT to suggest specific groups of PCRT neurons were preferentially targeted. Putative synaptic contacts were found between CEM axon terminals and infected PCRT neurons in the rostral cluster of tongue-related premotor neurons, the group of masseter-related premotor neurons dorsal to the facial nucleus, and with TA and IC related neurons in the PCRT dorsal to the nucleus ambiguus. For CEM axons as a whole, a parallel innervation pattern appears to be made in the PCRT. There are, however, two additional features: some groups of neurons in the PCRT are only very sparsely innervated by terminals from CEM axons while the axons themselves appear to pass through; and individual CEM axons may differ in which part of the PCRT they give off terminals. This latter observation may be linked with the finding that the distribution patterns of infected CEM neurons varied subtly depending on which muscle was inoculated.

CEM projections to the pons and medulla are largely, if not exclusively, GABAergic and form symmetrical synaptic contacts with all post-synaptic structures so far studied (Cassell and Gray, 1989, Pickel et al., 1995, Yasui et al., 2004). GABAergic mechanisms (Chen et al., 2001, Chen and Travers, 2003) play a critical role in switching between different oromotor behaviors, possibly through the adjustment of tonic inhibition, though emphasis has been given to GABA-containing neurons in the IRT with direct projections to moV and NXII (Travers et al., 2005). The PCRT/IRT act as an interface between the oral sensory inputs processed by the NTS and oro-pharyngeal motoneurons, generating rhythmic patterns as well as switching between complex stereotypic actions (Travers et al., 1997). GABAergic inputs to the PCRT/IRT from CEM could mimic or augment intrinsic GABA activity, switching, for example, between ingestion and rejection behaviors.

Taste aversion circuitry

The CE is a key node in the distributed network of structures involved in the acquisition and expression of conditioned taste aversion (CTA) in rodents (Yamamoto, 2007). In particular, lesions of the CE abolish the acquisition of a conditioned taste aversion, though only when the conditioning stimulus (CS) is introduced into the oral cavity and not when the animal is required to engage in instrumental behavior, such as approaching and drinking the CS from a bottle (Morris et al., 1999, Schafe et al., 2000). Studies examining activation of the immediate-early gene *cfos* in conditioned taste aversion show increased numbers of neurons containing Fos protein in CEM, CEL and the parvocellular basolateral nucleus, but not CELC or the lateral nucleus (Yasoshima et al., 2006). This suggests the circuit through CEM, CEL and the BLP defined here by PRV spread from oro-pharyngeal muscles via the pons and medulla may be the critical intra-amygdaloid circuit for CTA. In contrast, conditioned fear induces Fos expression in CELC, the magnocellular basolateral nucleus and the lateral nucleus (Scicli et al., 2004). Other portions of the extended amygdala complex such as the bed nucleus of the stria terminalis (BNST) and the interstitial nucleus of the

posterior limb of the anterior commissure (IPAC) have been implicated in CTA formation (Yasoshima et al., 2006). Though the BNST contained PRV infected neurons after oro-pharyngeal muscle injections – they were not systematically studied here – none were found in IPAC (FIGS2 and 3).

The present finding of CEM projections to high-order premotor neurons in the IRT as well as switchable central pattern generators for the oro-pharyngeal musculature suggests a role for this portion of the CE in the expression of conditioned taste aversions by engaging hard-wired circuits that generate responses to aversive intra-oral stimuli through gustatory input from the NTS. Large numbers of Fos expressing cells are found in the PCRT and IRT following intra-oral quinine (DiNardo and Travers, 1997) and heavy concentrations are located in the IRT and in the PCRT dorsal to the NA, both regions where CEM axons end (FIG. 7F and G). Intra-oral quinine appears not to induce Fos in the CE whereas application of a CS previously paired with quinine does (Yasoshima et al., 2006). This is consistent with a role for CEM in the expression of CTA.

Clearly, the trans-synaptic progression of PRV infection in the amygdala strongly suggests CEL became infected from CEM, consistent with a projection from CEL to CEM (Jolkkonen and Pitkanen, 1998) rather than direct connections between CEL and the pons (Petrovich and Swanson, 1997). Enhanced neuronal activity has been found in the CEL on presentation of a palatable conditioning stimulus (CS) previously paired with illness (US) induced by lithium chloride (Inui-Yamamoto et al., 2010) suggesting a role for CEL in acquisition and/or retrieval of the CS/US association. Intra-peritoneal administration of lithium chloride as part of CTA paradigms itself induces Fos strongly in CEL and much less so in CEM to (Yamamoto et al., 1997) pointing to potential CS/US associations in CEL (Yasoshima et al., 2006).

Conclusions

Retrograde trans-synaptic viral tracing from select oro-pharyngeal muscles has revealed a restricted set of amygdaloid nuclei connected through the central nucleus with clusters of ponto-medullary premotor neurons that may represent central pattern generators for stereotypic oro-pharyngeal movements. This restricted system, involving only the medial and lateral central nucleus, the parvicellular basolateral nucleus and the amygdalo-piriform transition area, may be the key amygdaloid circuit for conditioned taste aversion. If so, the lack of involvement of the lateral and magnocellular basolateral nuclei, and the shell or capsule of the central nucleus – all areas reportedly involved in fear conditioning – suggests distinct intra-amygdaloid circuits serve different modalities of aversive learning.

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ABBREVIATIONS

aca	anterior limb of anterior commissure
acp	posterior limb of anterior commissure
AHi	amygdalo-hippocampal transition area
APir	amygdalo-piriform transition area
AStr	amygdalo-striatal transition area
BDA	biotinylated dextran amine
BLM	magnocellular basolateral nucleus of amygdala

BLP	parvicellular basolateral nucleus of amygdala
BM	basomedial nucleus of amygdala
BNST	bed nucleus of the stria terminalis
BNSTpl	bed nucleus of the stria terminalis, posterolateral division
CA3	hippocampal subfield CA3
CE	central nucleus of amygdala
CEL	central nucleus, lateral subdivision
CELC	central nucleus, lateral capsular division
CEM	central nucleus, medial division
comNTS	commissural division, nucleus tractus solitarii
CP	caudate-putamen
CS	conditioning stimulus
CTA	conditioned taste aversion
DG	dentate gyrus
GABA	gamma amino-butyric acid
GG	genioglossus muscle
IBO	ibotenic acid
IC	inferior pharyngeal constrictor muscle
ICM	intercalated cell masses of amygdala
IPAC	interstitial nucleus of posterior limb of anterior commissure
IRT	intermediate reticular formation
LN	lateral nucleus of amygdala
MDd	dorsal medullary reticular nucleus
Me	medial nucleus of amygdala
MeV	medial nucleus, ventral division
mNTS	medial division, nucleus tractus solitarii
moV	motor nucleus of trigeminal nerve
MS	masseter muscle
NA	nucleus ambiguus
NAc	compact division, nucleus ambiguus
NAI	loose division, nucleus ambiguus
NAsc	semi-compact division, nucleus ambiguus
NTS	nucleus tractus solitarii
nVII	facial nerve
NVII	facial nucleus
NX	dorsal vagal motor nucleus

NXII	hypoglossal nucleus
ot	optic tract
PCA	posterior cricoarytenoid muscle
PCRT	parvicellular reticular formation
PCRTa	parvicellular reticular formation, pars alpha
PRV	pseudorabies virus
S	subiculum
SI	substantia innominata
st	stria terminalis
TA	thyroarytenoid muscle
US	unconditioned stimulus
v	lateral ventricle
VE_n	ventral endopiriform nucleus
vNTS	ventral division, nucleus tractus solitarii

FIGURE 1.

Horizontal sections through the medulla showing pseudorabies virus infected motor neurons in the motor trigeminal nucleus (A), hypoglossal nucleus (B), semi-compact (C) and loose (D) portions of the nucleus ambiguus 3 (A and B) or 4 (C and D) days following inoculation of the masseter, genioglossus, inferior pharyngeal constrictor and posterior cricoarytenoid muscles respectively. Scale Bar= 50 μ m.

FIGURE 2.

Horizontal sections through the amygdala showing the pattern of PRV infection in the central nucleus at 4 (panels A and C) and 5 days (B and D) after inoculation of the tongue (A and B) and masseter (C and D). Note the spread of PRV to neurons in the lateral subdivision (CEL) by 5 days p.i. but lack of infection in CELC and other nuclei of the amygdala. Sections shown in panels B and C were also immunochemically stained for calcitonin gene-related peptide which differentiates between CEM and CEL. Scale Bar=500 μ m.

FIGURE 3.

Horizontal sections through the amygdala showing the pattern of PRV infection in the central nucleus at 4 (panels A and C) and 5 days (B and D) after inoculation of the thyroarytenoid (A and B) and inferior constrictor C and D) muscles. As with tongue and masseter inoculations, infected neurons first appear in CEM followed by infection of CEL. A few infected cells can be seen the magnocellular basolateral nucleus (BLM) after thyroarytenoid inoculations in panels A and B but this was very exceptional. Scale Bar = 500 μ m.

FIGURE 4.

Distribution of PRV infected cells (black squares) in representative horizontal sections through the amygdala from animals euthanized at 4 (A) and 5 (B) days after inoculation of the virus into the inferior pharyngeal constrictor. Sections were 50 μ m thick and are spaced here approximately 200 μ m apart. Note the initial infection is confined to CEM and the parvicellular basolateral nucleus (BLP) and amygdalo-piriform transition area (APir). By 5 days, CEM, APir and BLP have many more infected neurons and CEL now contains infected neurons. Other amygdaloid nuclei, for example, the magnocellular basolateral (BLM), lateral (LN), basomedial (BM) and medial (Me, MeV) nuclei never or only rarely contained infected neurons. Scale Bar=2mm

FIGURE 5.

Horizontal sections (50 μ m thick, counterstained with cresyl violet) through the whole rat brain showing the location of sites in the reticular formation where ibotenic acid was injected and the resultant pattern of PRV spread 5 days after tongue and masseter inoculations. Infected motorneurons are indicated by arrowheads. Dashed circles indicate the extent of almost complete neuron loss and extensive gliosis. Ibotenic acid lesions involving the PCRT abolished (bold filled arrow, bottom panel) or severely attenuated (bold filled arrow, top panel) CE infection without major change in infection of the paraventricular (open arrows) and lateral hypothalamic nuclei. Injections of saline (small arrows in middle panel) into the PCRT had no effect on PRV spread to the forebrain. Scale Bar 5mm.

FIGURE 6.

A. Horizontal section (50 μ m thick, counterstained with cresyl violet) through the approximate mid dorsoventral level of the central nucleus showing the center of a cluster of BDA-filled neurons in CEM. The resulting terminal fields from this injection in the pons and medulla are shown in Figure 6. Scale Bar = 500 μ m. B and C. High power photomicrographs of CEM axons in 50 μ m thick sections through the intermediate (IRT) and parvocellular reticular formation. Note the long, sparsely branching axons in the IRT but the much more numerous side branches (arrowheads) in the PCRT (C). Scale Bar = 10 μ m. D. High power photomicrograph of a typical terminal efflorescence found at the end of side branches of CEM axons in the medulla. Though this image was of a side branch arising from

an axon (arrowhead) in IRT, most side branches of axons in PCRT had very similar terminal arrays. Scale Bar = 5 μ m.

FIGURE 7.

A–G. Dark field photomicrographs showing the distribution of BDA-labeled CEM axons and their branches in 50µm thick horizontal sections, 200µm apart, cut through the dorsal (A) to ventral (G) extent of the pons and medulla. Note the considerable size of the axon terminal field in the reticular formation which extends from ventral to the NTS (B) to surrounding the nucleus ambiguus (NA; F and G). Scale Bar= 500µm.

FIGURE 8.

Three-dimensional reconstruction of BDA-labeled CEM axons in the pons and medulla shown in a dorsal view (A) and a side view at an angle of 75° to the vertical (B). The position of the obex is marked by an 'x'. The ellipses on the left of the figure are the cross-sectional profiles of the facial nerve (nVII). In this case, 23 neurons in the CE were filled with BDA and all axons and branches were drawn on every 100µm thick section. Note the concentration of terminal bearing branches in four zones(1–4) in the parvicellular reticular formation and the substantial gap between zones 2 and 3 where mostly long axon segments are present. Scale Bar=1mm

FIGURE 9.

Reconstruction in the horizontal plane of two representative CEM axons in the pons and medulla. A. Axons were traced from two cells (arrowheads) in CEM labeled by a small deposit of BDA and their branches reconstructed from 150 μ m thick horizontal sections. B. Typical CEM axon that branched at the level of the descending limb of the facial nerve (nVII) sending branches to the IRT (arrowheads) and the ventral PCRT. This axon did not give rise to any branches in PCRT alpha (PCRTa). C. CEM axon that gave rise to branches with terminal boutons in the NTS (this segment shown separately in D), the IRT, PCRTa, and the ventral PCRT dorsal to the nucleus ambiguus (this segment shown separately in E). Scale Bar in A=500 μ m; in B–E = 1mm.

FIGURE 10.

Dorsal view of the pontine and medullary distributions of premotor neurons (small dots) in the IRT, PCRT and MDd infected by pseudorabies inoculation of the TA (A), IC (B), GG (C) and MS (D) muscles 4 days earlier. Large dots represent PRV infected motor neurons, and the distribution of laryngopharyngeal motor neurons is shown in the compact (NAc), semi-compact (NAsc) and loose (NAI) divisions of the nucleus ambiguus. The thick dashed line marks the boundary between the PCRT and IRT at roughly the mid-dorsoventral level of the pons and medulla. While there is considerable overlap in the caudal one-third of the respective distributions of premotor neurons, clear areas of non-overlap are evident in both IRT and PCRT. Note the rostral, non-overlapping clusters of premotor neurons infected

from tongue and masseter inoculations just caudal to the facial nerve; both these clusters appear to be in the zone 1 of concentrated CEM terminals shown in Figure 7. Scale Bar=1mm; divisions on side and top scales are 500 μ m.

FIGURE 11.

Lateral view of the pontine and medullary distributions of PRV infected premotor neurons (small dots) in the IRT, PCRT and MDd shown in Figure 10. Note that in addition to the mediolateral and rostrocaudal differences in distribution patterns shown in Figure 9, there are major differences in dorsoventral location of clusters of premotor neurons, particularly in the rostral two-thirds of the distribution.

FIGURE 12.

Horizontal sections of the brain showing the location of four representative BDA injections (arrows) made into the CE 7–10 days before inoculation of PRV into oro-pharyngeal muscles. The resulting interactions between BDA labeled terminals and PRV infected neurons are shown in Figures 12 and 13. Scale Bar=10mm.

FIGURE 13.

Putative contacts between CEM axon terminals and premotor neurons transneuronally labeled by PRV infection of the tongue (A–D) and masseter (E–G). A. Dark-field micrograph of horizontal section showing overlap between CEM axon terminals in zones 1, 2 and 3 and PRV-infected hypoglossal premotor neurons. Panels B–D show bright-field micrographs of the respective zones with a portion of putative contacts indicated (arrowheads). E. Dark field micrograph showing interactions between CEM axons and trigeminal premotor neurons. F and G. Bright field micrographs of the indicated areas. Scale Bar in A and E=200 μ m; in B–D and F and G=20 μ m.

FIGURE 14.

Putative contacts between CEM axon terminals and premotor neurons transneuronally labeled by PRV infection of the thyroarytenoid (A–D) and inferior constrictor muscle (E–H). A. Dark field micrograph of horizontal section showing putative contacts in zones 1 (B), 2 (C) and 4(D) between BDA-labeled axon terminals and vagal (ambiguous) premotor neurons related to the larynx. Panels B–D show bright field micrographs of the respective zones and putative contacts (arrowheads). E. Dark field micrograph showing putative contacts in zones 1 (B), 2 (C) and 4(D) between BDA-labeled axon terminals and vagal (ambiguous) premotor neurons related to the pharynx. Note that while CEM axon branches and terminals seem to be clustered in particular regions, contacts are made with premotor neurons related to all oro-pharyngeal muscles studied here. Scale Bar in A and E=200µm; in B–D and F and G=20µm.

FIGURE 15.

Horizontal sections through the intermediate reticular formation showing interactions between BDA-labeled CEM axons and PRV infected vagal premotor neurons following inoculation of the thyroarytenoid muscle. A. Clustering of premotor neurons in the IRT into groups of approximately 250–500 μ m diameter is evident. B and C show samples of putative contacts between CEM axon terminals and PRV infected neurons. Scale Bar in A=200 μ m, B and C=50 μ m.

Table 1
Pattern of infection of nuclei of the amygdala following inoculation of PRV into oro-pharyngeal muscles. Values represent the numbers of nucleated neuronal fragments >10µm in diameter found in a single horizontal section 50µm thick, except in the case of the lateral nucleus (LN) where the numbers represent the total number of fragments found in the whole nucleus.

INJECTION SITE	CEM	CEL	CELC	LN	BLP	BLM														
							i	c	i	c	i	c	i	c	i	c	i	c	i	c
PHARYNX																				
	1	8	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4 day	2	9	1	2	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0
	3	9	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
	1	101	53	43	6	1	1	0	0	61	47	9	3							
5 day	2	131	17	71	6	1	0	0	0	53	2	11	0							
	3	33	9	6	1	0	0	0	0	17	3	1	0							
TONGUE																				
	1	23	1	0	0	0	0	0	0	1	0	0	0							
4 day	2	51	1	4	0	1	1	0	0	1	0	0	0							
	3	13	0	0	0	0	0	0	0	0	0	0	0							
	1	132	92	63	31	7	2	9	3	69	18	22	5							
5 day	2	63	37	21	10	3	1	2	3	21	17	0	0							
	3	67	23	16	6	0	0	0	0	9	3	0	0							
MASSETER																				
	1	29	7	2	0	0	0	0	0	2	0	0	0							
4 day	2	18	4	0	0	0	0	0	0	0	0	0	0							
	3	36	2	5	0	0	0	0	0	0	0	0	0							
	1	103	79	27	13	2	0	0	0	13	12	0	0							
5 day	2	83	5	26	0	0	0	2	0	16	6	1	0							
	3	56	17	5	0	0	0	1	0	7	0	3	0							
LARYNX																				
	1	23	3	1	0	0	0	0	0	3	0	0	0							
4 day	2	9	1	0	0	0	0	0	0	0	0	0	0							

INJECTION SITE	CEM		CEL		CELC		LN		BLP		BLM	
	i	e	i	e	i	e	i	e	i	e	i	e
	3	19	2	1	0	0	0	0	3	0	0	0
	1	62	13	27	0	2	0	0	23	6	0	0
5 day	2	86	19	19	2	3	0	0	58	58	0	0
	3	91	10	17	3	3	0	0	9	0	3	0

Table 2

Effect of prior ibotenic acid lesions on infection of the ipsilateral (i) and contralateral (c) central nucleus by PRV inoculated into the tongue or masseter muscle. Numbers represent numbers of neuronal fragments >10µm in diameter found in a single horizontal section through the amygdala. The sampled section was at the same level as the section sampled in Table 1.

PRV INJECTION SITE	CEM	CEL	LESION		
	i	c	i	c	
MASSETER1(245)	9	3	1	0	Rostral; PCRT 0.5mm caudal to nVII; caudal: level of obex in dorsal PCRT
MASSETER2(246)	11	3	1	0	Dorsal and caudal 2/3rds PCRT; some caudal NTS involved
MASSETER 3(247)	21	7	5	0	Very similar to 246
MASSETER4(244)	67	11	6	0	Small in dorsal PCRT
TONGUE1(240)	3	1	0	0	2/3rds of PCRT from just caudal to nVII to level of obex
TONGUE2(242)	10	3	0	0	Very similar to case 240 except involvement of ventral NTS
TONGUE3(236)	0	0	0	0	Rostral:Caudal to nVII, about 1mm diameter; caudal: anterior to obex in PCRT
TONGUE 4(237)	19	29	9	0	Rostral: near nVII but small (0.25 mm diameter) in dorsal PCRT; caudal: caudal ½ of NTS and subjacent IRT