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Chapter 4

CONNECTIONS OF THE RODENT CENTRAL NUCLEUS OF AMYGDALA: A FUNCTIONAL VIEW

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ABSTRACT

The central nucleus of the amygdala (Ce) has connections with selective areas in the entire neuraxis. Through these projections, it receives a considerable amount of sensory information and is in the position to modulate somatic and autonomic responses to emotionally relevant stimuli. Its striatal-pallidal like organization may also contribute to the regulation of the "smoothness" and "tonus" of these emotional responses. Here, we give an overview of the connections of the Ce, including peptidergic connections, and present the organization of the projections of the Ce to four selected brain areas: the substantia innominata (SI), paraventricular nucleus of the hypothalamus (NPV), pontine nuclei in reticular formation (PNR) and dorsal motor nucleus of vagus/nucleus of the solitary tract (DMX/NST) complex. All four regions are involved in the modulation of distinct aspects of the emotional responses in the fear-sensitized acoustic startle reflex paradigm, in which they modulate arousal and attentional mechanisms, endocrine and cardiovascular stress responses, the startle response and parasympathetic functions. The contribution of subnuclei of the Ce to specific projections and their topography, as well as the occurrence of neurons that project to more than one target, are determined using

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the retrograde tracers Fluorogold (FG) and True Blue (TB). In addition, the proportion of neurons in the subnuclei of the Ce projecting to these areas is quantified. The timeline of various types of fear responses (fear-sensitized acoustic startle response, heart rate, blood pressure and body temperature) studied in a startle chamber and using telemetry electrodes are presented. The heaviest Ce projections are observed after injection into the DMX/NST, and the weakest projections after injections into the NPV. For all targets, the majority of Ce projection neurons (>60%) is located in the medial central nucleus (CeM). The lateral central nucleus (CeL) contributes to about 20% of the projections to the SI, about 10% to the DMX/NST, about 5% to the NPV and virtually none to the PNR. The contribution of the capsular part of the central nucleus (ClC) to projections is, in general, less than 5%. In contrast to the more dispersed distribution of neurons projecting to the SI, the neurons innervating the NPV, DMX/NST and PNR show a rather complementary arrangement, at least in the rostral and dorsal/dorsolateral parts of the CeM. The injection of both tracers in various combinations into the targets reveals in all cases double-labeled neurons, but in small numbers. The modality-specific input to subnuclei of the Ce and the organization of projections to the different downstream regions indicates that the Ce is more than a mere output relais of the amygdala. The differential organization of projections originating in the subnuclei of the Ce may be important for an independent treatment of various components of fear-, anxiety- and stress-related responses, because the brain nuclei examined are mainly targeted by separate populations of neurons within the Ce that may be recruited independently to regulate different aspects of emotional behavior.

Keywords: Fear, startle, emotion, arousal, stress, collateralization, topography, tracer

ABBREVIATIONS

AcB	accessory basal nucleus of the amygdala
ACo	anterior cortical nucleus of the amygdala
a-MSH	alpha-melanocyte stimulating hormone
ARC	arcuate nucleus of hypothalamus
BST	bed nucleus of stria terminalis
BSTL	bed nucleus of stria terminalis, lateral part
CCK	cholecystokinin
Ce	central nucleus of the amygdala
CeL	lateral subnucleus of the central nucleus of the amygdala
CeM	medial subnucleus of the central nucleus of the amygdala
CGRP	calcitonin gene-related peptide
ClC	capsular subnucleus of the central nucleus of the amygdala
cNST	commissural nucleus tractus solitarius
CP	caudate-putamen
CRF	corticotropin-releasing factor
DMX	dorsal vagal motor nucleus (or complex)
dRN	dorsal raphe nucleus
DYN	dynorphin
ENK	enkephalin
GAD	glutamic acid decarboxylase

GAL	galanin
GP	globus pallidus
HYP	hypocretin (also called orexin)
III	third ventricle
LC	locus coeruleus
ldTG	latero-dorsal tegmental nucleus
LENK	leucine-ENK
LH	lateral hypothalamus
Me	medial nucleus of the amygdala
MENK	methionine-enkephalin
MNRpv	reticular nucleus of medulla oblongata, parvocellular part
mRF	mesencephalic reticular formation
mV	mesencephalic nucleus of the trigeminal nerve
NPV	paraventricular nucleus of hypothalamus
NPVpv	paraventricular nucleus of hypothalamus, parvocellular part
NPY	neuropeptide Y
NST	nucleus of the solitary tract
NT	neurotensin
NTS	nucleus of the solitary tract
OPC	opiocortin
PAG	mesencephalic periaqueductal (central) gray
PBN	parabrachial nuclei of pons
PEMT	phenylethanolamine-N-methyl-transferase
pFX	perifornical nucleus of hypothalamus
PNR	pontine reticular nucleus
pTH	posterior thalamic complex
PVTh	paraventricular nucleus of thalamus
pyr	pyramidal tract
SI	substantia innominata
SMN	supramammilary hypothalamic nucleus
SN	substantia nigra
SOM	somatostatin
SP	substance P
ST	stria terminalis
TH	tyrosine hydroxylase
TIP39	tuberoinfundibular peptide of 39 residues
TRH	thyrotropin-releasing hormone
V	spinal trigeminal nucleus
VII	facial nerve
VIP	vasoactive intestinal peptide
VLM	ventrolateral medulla
vmGP	globus pallidus, ventromedial part
VMN	ventromedial hypothalamic nucleus
VTA	ventral tegmental area

1. INTRODUCTION

A variety of behavioral, physiological and anatomical studies provide evidence that the central nucleus of the amygdala (Ce) plays an important role in the regulation of autonomic, endocrine and involuntary somatomotor components of conditioned emotional responses (rev. in Davis, 1992; Gallagher and Holland, 1992). The anatomical basis for most of these responses is well studied in mammals, e.g., in rodents the Ce may modulate directly, via its projection to the pontine reticular formation (PNR), the acoustic startle reflex (Rosen et al., 1991) and nictitating membrane reflex in rabbits (Whalen and Kapp, 1991). Projections to periaqueductal gray are important for changes in locomotor activity, freezing and defensive responses (Rizvi et al., 1991; Walker et al., 1997). Pathways implicated in the activation of the hypothalamic-pituitary-adrenal (HPA) axis and stress-responses are direct and/or indirect projections of the Ce to hypothalamic regions, the locus coeruleus and the nucleus of the solitary tract (NTS)-dorsal motor nucleus of vagus (DMX) complex (Danielsen et al., 1989; Gray et al., 1989; Loewy, 1990; Petrovich et al., 2001; Curtis et al., 2002; Nunn et al., 2011). Among these targets, the hypothalamic paraventricular nucleus (NPV) is the origin of central sympathetic efferents (Nunn et al., 2011). Through its projections to the NTS/DMX complex, the Ce can also directly influence the incoming sensory information from the viscera and the output signals of the DMX (Saha et al., 2000; Saha, 2005). In addition to these widespread brainstem and diencephalic projections, a heavy projection is directed toward the substantia innominata (SI) (Grove, 1988b; Jolkkonen et al., 2002), through which the Ce may influence cortical activity by acting directly or indirectly on cholinergic neurons of the magnocellular basal forebrain complex (Dringenberg and Vanderwolf, 1996).

Although these projections of the Ce are well-characterized, it is less clear how the Ce orchestrates these various aspects of emotional behavior. For example, a loud unexpected noise induces a simultaneous twitch of somatic muscles known as the acoustic startle response, activates the cardiovascular system and increases the attentional level; all these reactions are modulated by the Ce (LeDoux et al., 1988; Yeomans and Frankland, 1995; Lee et al., 1996; Koch and Schnitzler, 1997; Saha et al., 2005). The concurrence of these actions and the widespread projections of the Ce create the challenging question of how the output of the Ce is topographically organized, e.g., whether separate populations of neurons are intermingled in the Ce, or clusters of arranged sets of neurons are responsible for the behavioral responses. Data from the efferent projections from several nuclei of the amygdala, e.g., the medial and basal nucleus, revealed that these projections are often topographically arranged (McDonald, 1991b; Canteras et al., 1995). A further possibility for the arrangement of Ce projection systems is that collaterals of single neurons innervate two or more targets in parallel and modulate different functions at the same time. This was shown for the projection of the basal nucleus to the frontal cortex and the striatum (McDonald, 1991a), and for an individual neuron in the lateral amygdaloid nucleus that possessed axon terminals in the entorhinal cortex, the postrhinal cortex and the amygdalo-striatal transition zone (Pitkänen et al., 2000). In a preliminary study (Fritz et al., 2005), we have demonstrated that parallel innervation of two distant targets, such as the substantia innominata and the pontine reticular formation, by single neurons occurs but is extremely rare, although separate sets of neurons in the Ce provide substantial input to both areas.

Here we will give an overview of the functional connections of the Ce and present the topography of projection neurons innervating four target areas. These targets, namely the SI, NPV, PNR and DMX/NTS complex, were selected on the basis that they are located within different brain areas but involved in responses to emotionally relevant and/or loud acoustic stimuli. For all of these target areas, the connections from the Ce are well-documented with both anterograde and retrograde tracing techniques, showing projections to the SI (Grove, 1988b; Jolkkonen et al., 2002), NPV (Gray et al., 1989; Prewitt and Herman, 1998; Petrovich et al., 2001), PNR (Hopkins and Holstege, 1978; Rosen et al., 1991) and DMX/NTS complex (Schwaber et al., 1982; van der Kooy et al., 1984; Wallace et al., 1992; Pickel et al., 1995; Liubashina et al., 2000; Saha et al., 2000). The present study was undertaken to examine whether targets of the Ce other than the PNR and SI are also innervated by separate neuronal populations in the Ce, and how the topography of projections to these targets is organized.

2. DIVISIONS OF THE CENTRAL NUCLEUS OF AMYGDALA (CE)

2.1. Cytoarchitectonics

The Ce is an ovoid cell mass within the dorsocentral part of the amygdala that is bordered laterally by the lateral amygdaloid nucleus, medially by the medial amygdaloid nucleus and dorsally by the globus pallidus (de Olmos et al., 2004). In Nissl (cresyl violet)-stained sections, three subnuclei are readily distinguished: a centrolateral subnucleus (CeL), a centromedial subnucleus (CeM) and a centrocapsular subnucleus (ClC) (see Figure 1). Additionally, a ventral centromedial (Cassell et al., 1986) and intermediate subnucleus were described, the latter discernible in Calbindin-dk28 stained sections (McDonald, 1997), which was also reported to have specific downstream connections (Cassell et al., 1999). We will adhere here mainly to the commonly used tripartition of the Ce (e.g., Swanson, 1992; Bernard et al., 1993; Cassell et al., 1999) and refer to the potential site of the central intermediate and centroventral subnuclei in our sections, where appropriate. The CeM is a comet-like cell mass with its head rostral and its tail reaching caudally to the level where the stria terminalis leaves the Ce. The neurons are ovoid, fusiform or piriform with a diameter of 10-20 µm. The CeL can be clearly delineated from the CeM due to its round shape and more intense staining of Nissl substance. In the rostrocaudal axis, this subnucleus displaces the CeM and is responsible for the comet-like tail. Neurons of the CeL are rather round, with a diameter of 12-16 µm (McDonald, 1992). The ClC is situated between the basolateral complex and the CeL. Rostrally, the ClC is attached directly to the CeM, whereas caudally, the ClC forms a dorsal and ventral mass that is connected by a thin cell-poor stripe of tissue that also extends laterally, where it borders the amygdalo-striatal transition zone.

2.2. Neuronal Cell Types

In the mammalian Ce studied in rats, cats and dogs, the morphology of neurons in the CeL and ClC differs from neurons in the CeM. The CeL and ClC contain medium-sized spiny neurons with extensively branching dendrites and high spine densities resembling striatal

neurons. In contrast, the principal neurons of the CeM often have ovoid or fusiform perikarya with two to four primary dendrites and a few higher order branches that are covered with a moderate-to-sparse number of spines (McDonald, 1992). Morphologically identified principal neurons were shown to express GABA and peptides and possess typical electrophysiological features in the rat Ce (Sun and Cassell, 1993; Veinante et al., 1997; Schiess et al., 1999).

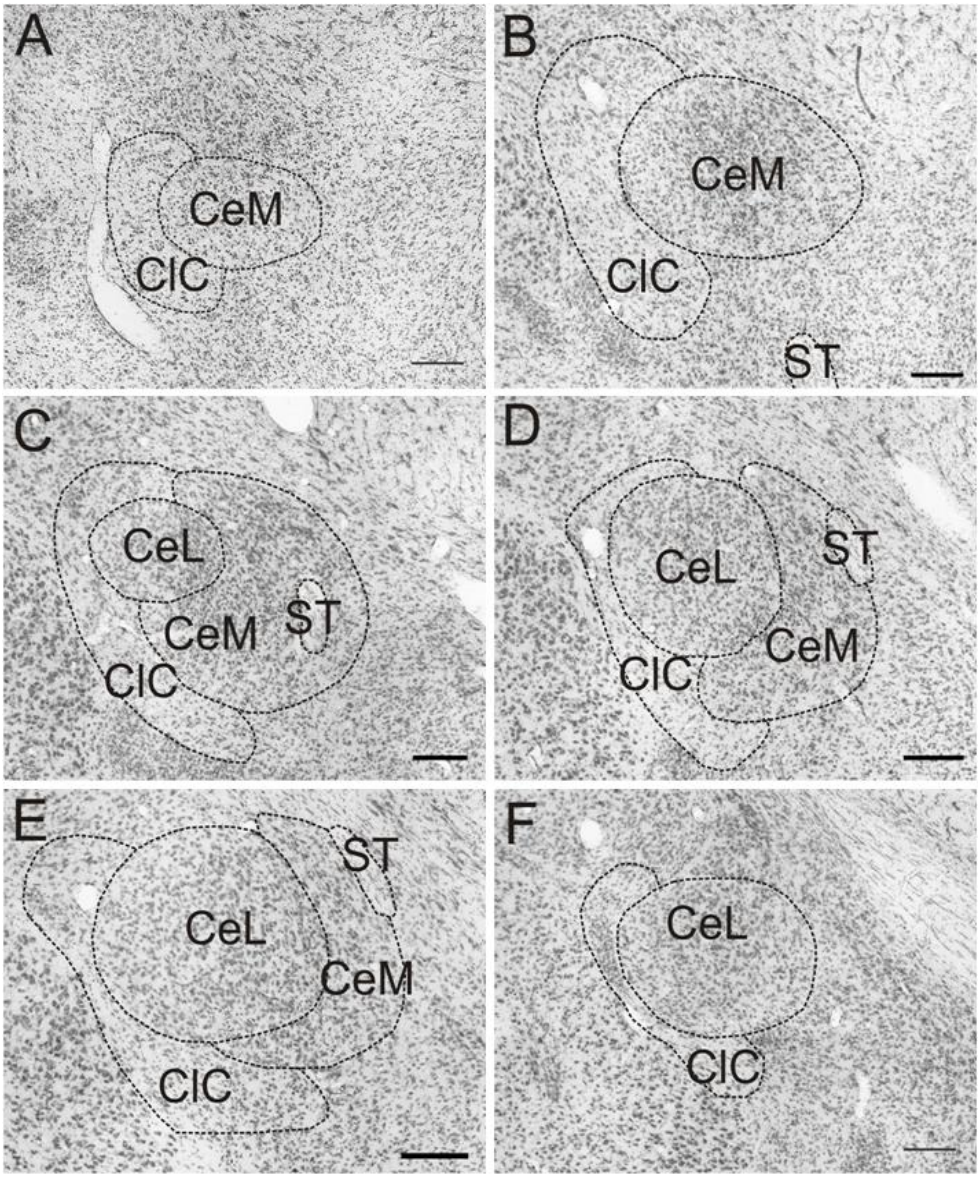


Figure 1. Cytoarchitecture of the central nucleus of the rat amygdala (Ce) from rostral (A) to caudal (F) at six representative levels. Sections are spaced between 200-400µm each. Rostrally (A, B) the Ce is composed only of the medial and capsular parts. When the stria terminalis appears (C), the lateral subnucleus forges between the capsular and medial subnucleus and displaces the medial subnucleus (F). CeM, medial subnucleus of the Ce; ClC, capsular subnucleus of the Ce; CeL, lateral subnucleus of the Ce; ST, stria terminalis. Calibration bars = 200µm.

In addition to the spiny principal neurons, the various divisions of the Ce also contain a small number of spine-sparse interneurons thought to be interneurons (McDonald, 1992). Among those, large neurons with smooth dendrites seem to resemble large cholinergic interneurons of the striatum, as indicated by electrophysiological studies, where they showed a depolarized resting membrane potential, and a long slow-after-hyperpolarization accommodating response in the rat amygdala (Schiess et al., 1999).

2.3. Developmental Origin

According to the concept of the extended amygdala, the Ce shares similarities with the lateral part of bed nucleus of the stria terminalis (BST) and sublenticular cell groups in the SI (Heimer et al., 1997; de Olmos et al., 2004), and recently it has been suggested that the intercalated nuclei (ICN) should also be included into this group (Yilmazer-Hanke, 2012). Neurons in the lateral part of the extended amygdala (including intra-amygdaloid division) originate mostly from the striatal primordium, and thus express markers of the lateral ganglionic eminence. Only the BST displays neurons with striatal markers at later stages of development, suggesting that it is populated by inwandering neurons (Nery et al., 2002; García-López et al., 2008).

However, there are also a few exceptions to this rule, as can be predicted from the original concept of Cassell and colleagues (1999), who proposed that the three main subnuclei of the Ce share features either with the shell or core of the accumbens or ventral pallidum (Figure 3 in Cassell et al., 1999). Recent developmental data show that most neurons of the CeL and ClC indeed originate from the striatal primordium, whereas the CeM is composed of a fairly mixed neuronal population. Thus, the CeM contains cells derived from striatal primordium, as well as pallidal-like neurons that originate from the anterior peduncular area and commissural neurons from the preoptic area. Moreover, developmental data indicate that Ce neurons originating from subpallial areas are probably GABAergic (Nery et al., 2002; García-López et al., 2008; Hirata et al., 2009).

In addition, subsets of peptidergic neurons in the Ce originate from specific subregions of the ganglionic eminences, e.g., islet1-expressing CRF-positive neurons seem to be born in the ventral part of the lateral ganglionic eminence, whereas SOM-positive neurons derive from the ventrocaudal part of the medial ganglionic eminence (Bupesh et al., 2011).

3. Overview of Connections

3.1. Input to the Ce

As mentioned above, the Ce is classically regarded as a motor output station of the amygdala, but often it is not considered that most of its downstream connections are reciprocal. From a functional point of view, it is important to mention that the Ce can respond to sensory stimuli, as was shown for noxious stimuli (Bernard et al., 1992), and it receives considerable input from sensory or sensory-related areas.

Thus, the Ce is innervated by the spinal cord (ipsi- and contralateral) and brainstem areas receiving sensory input, namely the dorsolateral pons and parabrachial nuclei (Saper and Loewy, 1980; Block et al., 1989; Burstein et al., 1991; Bernard et al., 1993; Newman et al., 1996). Projection neurons targeting the Ce are found throughout the length of the spinal cord (bilaterally) and are located mainly in the lateral dorsal horn, pericentral laminae (around the central canal) and lateral spinal nucleus, although many neurons seem to be concentrated in upper lumbar and upper cervical segments (Burstein and Potrebic, 1993; Newman et al., 1996). Moreover, the innervation of the Ce by the pontine parabrachial nucleus is topographically organized (Yamano et al., 1988a; 1988b; Block et al., 1989; Bernard et al., 1993), i.e., the parabrachial input to the CeM is mainly gustatory; to the CeL visceral and chemosensitive; and to the ClC nociceptive, respiratory and cardiovascular (Bernard et al., 1993). Among mesencephalic areas, the periaqueductal gray (Ottersen, 1981; Li et al., 1990; Hurley et al., 1991; Rizvi et al., 1991), retrorubral field and cuneiform nucleus (80% from noncholinergic neurons) (Hallanger and Wainer, 1988) and laterodorsal tegmental nucleus (Nitecka et al., 1980) also provide input to the Ce.

Hypothalamic areas provide substantial input to the Ce; the purpose of these projections may be to provide feedback information to the Ce that regulates the activity of the hypothalamus for mediating autonomic and stress responses (Ulrich-Lai and Herman, 2009). However, many hypothalamic regions projecting to the amygdala (e.g., lateral hypothalamus, posterior and dorsal hypothalamic areas, dorsomedial nucleus, lateral and medial preoptic areas) also receive afferents from sensory regions themselves (Cliffer et al., 1991), and it is not known whether these areas function as relay stations providing sensory input to the Ce. The heaviest hypothalamic projections to the Ce originate in the lateral hypothalamus, ventromedial nucleus and premammillary nucleus, but the posterior hypothalamus, arcuate nucleus and dorsomedial nucleus also innervate the Ce, and many of these projections target the CeM and ClC (Post and Mai, 1978; Veening, 1978; Krieger et al., 1979; Ottersen, 1980; Canteras et al., 1992; Canteras et al., 1994; Vertes et al., 1995; Thompson et al., 1996). In addition, the Ce receives moderate-to-light projections from the anterior hypothalamic area, preoptic area, retrochiasmatic nucleus and perifornical area (Swanson, 1976; Ottersen, 1980; Nitecka, 1981; Risold et al., 1994).

The posterior paralaminar complex of the thalamus, known to be important for sensory integration, also sends a prominent input to the CeM, but largely spares the CeL (Linke et al., 2000; D'Hanis et al., 2007). Thus, the CeM is innervated by the peripeduncular nucleus (Nitecka et al., 1979; Ottersen and Ben-Ari, 1979; Linke et al., 2000) involved in acoustic functions (Bordi and LeDoux, 1992; Linke, 1999); the posterior intralaminar thalamic nucleus (LeDoux et al., 1990; Linke et al., 2000) that receives somatosensory, acoustic and nociceptive information (Bordi and LeDoux, 1994a; 1994b; Linke, 1999; Shi and Davis, 1999); and the subparafascicular thalamic nucleus (Veening, 1978; Nitecka et al., 1979; Ottersen and Ben-Ari, 1979; D'Hanis et al., 2007), which contributes to auditory, nociceptive and visceral information processing (Gaytan and Pasaro, 1998; Coolen et al., 2003a; 2003b; Wang et al., 2006). Furthermore, the medial part of the medial geniculate body and posterior intralaminar thalamic nucleus send projections to the lateral-most zone that includes the ClC and amygdalo-striatal transition area (Veening, 1978; Ottersen and Ben-Ari, 1979; Turner and Herkenham, 1991; Linke et al., 2000). Projections from the visually driven suprageniculate nucleus were also reported (Nitecka et al., 1979), but the latter input seems to be rather weak (Linke et al., 2000). Nakashima et al. (2000), however, demonstrated a rather precise

projection from the VPMpc to the CeL. Other thalamic afferents arising in the sensory ventral posterior nucleus (including the parvicellular ventroposterior nucleus, the thalamic taste relay) also reach the Ce, and the taste pathway seems to specifically target the CeM (Ottersen and Ben-Ari, 1979; Turner and Herkenham, 1991). In addition, the midline thalamic nuclei are connected intensely with the Ce, i.e., the Ce receives substantial input from the paraventricular thalamic (mainly CeL), reuniens, centromedian and paratenial nuclei (Krettek and Price, 1978; Veening, 1978; Nitecka et al., 1979; Ottersen and Ben-Ari, 1979; Kelley and Stinus, 1984; Turner and Herkenham, 1991; Moga et al., 1995; Vertes and Hoover, 2008).

As reviewed thoroughly by McDonald (1998) and Pitkänen (2000), the Ce further receives input from a large number of sensory-related cortical areas, although some of these projections may also be involved in regulating the motor functions of the Ce. Interestingly, the modalities of sensory-related cortical inputs mostly resemble the subcortical inputs described above, suggesting a high degree of convergence of modality-specific information onto individual subnuclei of the Ce. For example, the ClC receiving projections from nociceptive subcortical areas is heavily innervated (in its rostral part) by the somatosensory parietal cortex (SII). In contrast, the visceral and gustatory parts of the dysgranular insula provide a very strong input to the CeL, and a light-to-moderate input to the CeM, whereas the posterior agranular insula (pressor responses) targets all portions of the Ce. Other cortical inputs to the Ce arise in the dorsal agranular insula, as well as auditory temporal association cortex TE2 (Yasui et al., 1991; Mascagni et al., 1993; McDonald and Mascagni, 1996; McDonald, 1998; Shi and Cassell, 1998). The piriform cortex only sends light projections to the CeL/ClC; however, the ClC receives considerable innervation from the lateral entorhinal and dorsal perirhinal cortices and the hippocampal system (ventral subiculum), whereas the CeM is innervated specifically by the lateral but not medial part of the ventral subiculum (Ottersen, 1982; Canteras and Swanson, 1992; Petrov et al., 1994; McDonald and Mascagni, 1997; McDonald, 1998). Projections from the prefrontal cortex originate in infralimbic and prelimbic areas known to have opposing roles in the expression of fear and drug seeking (Ulrich-Lai and Herman, 2009), but the infralimbic area targets the ClC and rostroventral portions of the CeM, whereas the prelimbic area sends a heavy projection largely confined to the ClC (Ottersen, 1982; Cliffer et al., 1991; Hurley et al., 1991; Katter et al., 1991; McDonald et al., 1996).

Diffuse corticopetal projecting systems often have reciprocal connections with the Ce. The Ce receives input from the substantia innominata and various cholinergic nuclei, i.e., the nucleus of the diagonal band, pedunculopontine tegmental nucleus and laterodorsal tegmental nucleus (Ottersen, 1980; Luiten et al., 1985; 1987; Hallanger and Wainer, 1988; Grove, 1988a; Petrov et al., 1994), but cholinergic fibers are largely confined to the CeM and ClC (de Olmos et al., 2004). The Ce is further innervated by the tuberomammillary hypothalamic nucleus containing histaminergic neurons (Airaksinen et al., 1989), as well as the zona incerta (A13 group), substantia nigra pars compacta and ventral tegmental area harboring dopaminergic neurons (Beckstead et al., 1979; Ottersen, 1981; Oades and Halliday, 1987; Seroogy et al., 1989; Wagner et al., 1995; Cheung et al., 1998). Monoaminergic fibers were reported to arise in the serotonergic dorsal raphe, noradrenergic locus coeruleus and A2 group, and adrenergic C1 group in the brainstem (Fallon et al., 1978; Veening, 1978; Nitecka et al., 1980; Ottersen, 1981; Li et al., 1990; Zardetto-Smith and Gray, 1990; Rizvi et al., 1991; Vertes, 1991; Petrov et al., 1994; Zardetto-Smith and Gray, 1995).

3.2. Intra-Amygdaloid Connections, Extended Amygdala and Microcircuitry

The rodent Ce receives light projections from the ipsilateral lateral amygdaloid nucleus and moderate-to-heavy projections from the ipsilateral basal, accessory basal, anterior cortical and medial amygdaloid nuclei, which innervate mainly the ClC and CeM, but do not provide substantial projections to other amygdaloid nuclei. In addition, the basal and anterior cortical, as well as the nucleus of the lateral olfactory tract, also seem to send projections to the contralateral Ce (Pitkänen, 2000). However, the input from the GABAergic intercalated nuclei of the amygdala reaches both the CeL and CeM, which is important for the modulation of the amygdaloid output through disinhibitory mechanisms (Royer et al., 1999). For the same reason, the connections between the subnuclei of the Ce also attracted attention. Whereas the CeL projects to the ClC and CeM, and the CeM also receives afferents from the ClC, the CeL does not receive any input from the other subnuclei of the Ce (Jolkkonen and Pitkänen, 1998). Interestingly, the Ce can also inhibit its own action, because the axons of projection neurons in the Ce possess feedback collaterals (rev. in McDonald, 1992). In addition, the Ce is reciprocally connected mainly with the lateral part of the BST (Krettek and Price, 1978; Weller and Smith, 1982; Sun et al., 1991; Petrovich and Swanson, 1997; Prewitt and Herman, 1998; Dong et al., 2001).

3.3. Output of the Ce

Among all amygdaloid nuclei, the Ce has the most widespread downstream projections, although the rodent Ce provides only a small amount of reciprocal input to cortical areas or cortex-like nuclei of the amygdala (Pitkänen, 2000; de Olmos et al., 2004). In addition to the intense connections between the Ce and BST described above, the Ce also innervates the lateral septum and substantia innominata (Grove, 1988b; Calderazzo et al., 1996; Petrovich and Swanson, 1997). There is considerable evidence that the Ce is the motor output center of the amygdala (e.g., Ulrich-Lai and Herman, 2009), but for understanding motor functions regulated by the Ce, it is important to realize that individual subnuclei of the Ce have similarities with specific striato-pallidal areas(Cassell et al., 1999), although the subnuclei of the Ce and striato-pallidal areas clearly differ in their downstream targets and functions (Zahm et al., 1999). Most of the downstream projections of the Ce originate in the CeM, and in part also in the ClC (e.g., projections to hypothalamus), whereas the CeL seems to have three major target areas, namely the CeM, BST and pontine parabrachial nucleus (Petrovich and Swanson, 1997; Pitkänen, 2000).

Based on connectional and neurochemical data, it has been suggested that the ClC resembles the shell and the CeL the core of the accumbens, whereas the CeM shares features with the ventral pallidum (Figure3 in Cassell et al., 1999), which may be important for regulating the "smoothness" or "tonus" of motor functions regulated by the Ce, or may even modulate the function of the dorsal striatum through amygdalo-nigro-striatal pathways (Han et al., 1997). This hypothesis is in agreement with the innervation of the "pallidum-like" CeM, globus pallidus and pars reticulata of the substantia nigra (considered functionally related to pallidum) by the "striatum-like" CeL/ClC (Schmued et al., 1989; Shinonaga et al., 1992; Petrovich and Swanson, 1997; Jolkkonen and Pitkänen, 1998), whereas projections of the Ce reaching the mediodorsal thalamic nucleus originate mainly from the "pallidum-like"

caudal CeM (McDonald, 1987; Cornwall and Phillipson, 1988; Vankova et al., 1992). Furthermore, the CeL and CeM innervate dopaminergic neurons in the substantia nigra (Post and Mai, 1980; Schmued et al., 1989; Wallace et al., 1989; Gonzales and Chesselet, 1990; Vankova et al., 1992; Cassell et al., 1999) comparable to the organization of striatonigral and pallidonigral projections, respectively (rev. in Tepper and Lee, 2007); the Ce also innervates (together with the globus pallidus) the subthalamic nucleus (Schmued et al., 1989). In contrast, projections of the Ce to the midbrain ventral tegmental area (A10 group) are comparatively light compared to the accumbens (Wallace et al., 1989; Zahm et al., 1999), a system associated with reward-related behavior (Yilmazer-Hanke, 2008). The Ce further provides input to midline thalamic nuclei, among which the paraventricular nucleus receives the strongest projection, followed by the rhomboid nucleus, although projections to the centromedian nucleus were also reported. In addition, the Ce sends efferents to the habenula, the rostral and ventral sectors of the zona incerta and the gustatory thalamic region (Vankova et al., 1992; Pitkänen, 2000).

The Ce innervates several hypothalamic areas, and many of these projections seem to arise in the CeM. The lateral hypothalamus and perifornical area implicated in various functions, including the regulation of feeding, sleep and wakefulness, and autonomic functions, including conditioned cardiovascular responses (Iwata et al., 1986; Oades and Halliday, 1987; Mihailoff et al., 1989; Bonnavion and de Lecea, 2010; Berthoud and Munzberg, 2011), receive the heaviest projections from the CeM, although some projections also originate from the CeL (Krettek and Price, 1978; Ono et al., 1985; Prewitt and Herman, 1998; Petrovich et al., 2001). The intensity of projections from the Ce to the hypothalamic NPV and the impact of these projections on the activation of the HPA axis are controversial, probably due to the variability in injection sites, including the CeL, although the projections originate mostly in the CeM (Gray et al., 1989; Gray, 1990; Prewitt and Herman, 1998; Petrovich et al., 2001; see also results in this chapter). The Ce further sends light projections to the anterior hypothalamic area and dorsomedial hypothalamic nucleus (Ono et al., 1985; Gray et al., 1989; Prewitt and Herman, 1998; Petrovich et al., 2001).

One of the major motor outputs of the Ce is directed toward the midbrain periaquaductal gray (Post and Mai, 1978; Post and Mai, 1980; Beart et al., 1990), which is known to be organized in longitudinal columns with specific functions, i.e., regulation of aversive/ defensive reactions versus fight/flight reactions, sexual posture and antinociception (Bandler and Keay, 1996; Van Bockstaele et al., 1996; Walker et al., 1997). The projections of the Ce originate mainly in the CeM and target the dorsomedial column and lateral/ventrolateral columns (behavioral and autonomic components of the defense/aversion responses) of the periaqueductal gray, also called the central gray (Rizvi et al., 1991). Other brainstem areas receiving projections from the Ce include the mesencephalic nucleus of the trigeminal nerve and the pontine parabrachial nuclei (Post and Mai, 1980; Gray, 1990; Sun et al., 1994; Petrovich and Swanson, 1997). The Ce further sends efferents to cholinergic/non-cholinergic neurons in the laterodorsal and pedunculopontine tegmental nuclei and dorsal vagal complex (dorsal motor nucleus of vagus, ambiguus nucleus, nucleus of the solitary tract) and monoaminergic/non-monoaminergic neurons in the dorsal raphe (serotonergic), raphe magnus/pallidus (serotonergic), gigantocellular reticular nucleus (pars alpha, serotonergic), retrorubral field (A8 dopaminergic cells), locus coeruleus (rostral A6 noradrenergic cells), subcoeruleus, nucleus of the solitary tract (C2 adrenergic and A2 noradrenergic cells) and rostral ventrolateral medulla (C1 adrenergic cells) (Post and Mai, 1980; Veening et al., 1984;

Danielsen et al., 1989; Schmued et al., 1989; Thompson and Cassell, 1989; Wallace et al., 1989; Rosen et al., 1991; Semba and Fibiger, 1992; Wallace et al., 1992; Pickel et al., 1995; Van Bockstaele et al., 1996; Hermann et al., 1997; Petrovich and Swanson, 1997; Lee et al., 2007). Projections from the Ce to the cuneiformis nucleus and basilar pontine nuclei, caudal pontine reticular nucleus (important for the startle response) and medullary reticular formation were also described (Bernard et al., 1989; Mihailoff et al., 1989; Rosen et al., 1991).

4. PEPTIDERGIC CONNECTIONS

The peptidergic connections of the amygdala will be presented separately, because it is not clear whether all Ce neurons are indeed peptidergic, i.e., co-express one or more peptides together with glutamic acid decarboxylase (GAD), the key enzyme involved in GABA synthesis. If such neurons exist, they would be "GABAergic only" neurons in the Ce. Lack of GAD (although not all isoforms studied) in some peptidergic neurons in the Ce suggests that there may be also "peptidergic only" neurons devoid of GABA, but if these neurons exist, they are probably rare (Veinante et al., 1997; Poulin et al., 2008). The third type of neuron co-expresses GAD with peptides, e.g., GAD expression has been reported in corticotropin-releasing factor (CRF)-, neurotensin (NT)- and methionine-enkephalin (MENK)-containing neurons in the Ce. However, CRF and NT were co-expressed in these neurons, whereas CRF and MENK were never expressed together in the same neuron (Veinante et al., 1997). Developmental data show that subsets of neurons expressing certain peptides in the Ce can differ in their origin, e.g., they can derive from different subpallial regions (Bupesh et al., 2011). Furthermore, neurons co-expressing specific combinations of peptides often target different downstream brain regions (Table 1), and are distributed over an area that is not restricted to a single subnucleus of the Ce, indicating that neurons migrating to the Ce during development do not always respect the boundaries between its subnuclei (e.g., Veening et al., 1984; Moga and Gray, 1985).

4.1. Peptidergic Input

The Ce is among the brain regions that receives the richest peptidergic innervation. The distribution pattern of peptidergic fibers, often colocalizing acetylcholine or monoamines in the Ce, can be used to identify chemoarchitectonic divisions, helping to delineate the subnuclei of the Ce (Table 1).

The pattern of peptidergic fibers also gives a first impression of fiber systems preferentially regulating the function of specific subnuclei of the Ce, or even compartments within individual subnuclei (e.g., de Olmos et al., 2004).

Table 1. Peptidergic input to the central nucleus of the amygdala (Ce)

Input to Ce from	Me, AcB, ACo	BSTL	pTH	SMN	LH	pFX	VMH	ARC	NPVpv	PAG	SN, VTA	dRN	PBN	ldTG	NST	cNST	VLM
	Amygdala and Extended amygdala		Thalamus	Hypothalamus						Brainstem							
a-MSH								21 ?									
CCK										32	32						
CGRP			8										8,31,33				
CRF					35							35					
DYN					41	41							7		28		
ENK	26	26					26						26		42		
HYP					25	25											
LENK										19							
MENK															28		
NPY															28,42		43
NT										19			6,39		28,42		
OPC																34	
SOM															28		
SP													6,40	24			
TIP39			9														
TRH									38								
VIP				5	5												

For abbreviations used in the table, see List of Abbreviations. References are numbered as follows: 5. Baek et al., 1988; 6. Block et al., 1989; 7. Code and Fallon, 1986; 8. D'Hanis et al., 2007; 9. Dobolyi et al., 2003; 19. Li et al., 1990; 21. O'Donohue and Jacobowitz, 1980; 24. Petrov et al., 1994; 25. Peyron et al., 1998; 26. Poulin et al., 2006; 28. Riche et al., 1990; 31. Schwaber et al., 1988; 32. Seroogy et al., 1989; 33. Shimada et al., 1985; 34. Sim and Joseph, 1994; 35. Uryu et al., 1992; 38. Wittmann et al., 2009; 39. Yamano et al., 1988a; 40. Yamano et al., 1988b; 41. Zardetto-Smith et al., 1988; 42. Zardetto-Smith and Gray, 1990; 43. Zardetto-Smith and Gray, 1995.

Table 2. Peptidergic output of the Ce

Output of Ce	BSTL	vmGP	PVTh	LH	PAG	mRF	mV	SN	VTA	PBN	dRN	ldTG	NST	DMX	LC	VLM	PNR	MNRpv
	Extended Amygdala	Pallidum	Thalamus	Hypothalamus	Brainstem													
CRF	16,31		22	16,30	15,16	30	30		29	16,20, 23,30, 37			16	14,16, 37	18		10	
DYN								36										
ENK	4	3																
GAL					13													
LENK	27																	
MENK								36										
NPY	2																	
NT	4			1	15			12, 26		20,37				14,37				
SOM					15					20,23, 37			17	14,17, 37				
SP	4				15									14				11

For abbreviations used in the table, see List of Abbreviations. References are numbered as follows: 1. Allen and Cechetto, 1995; 2. Allen et al., 1984; 3. Arluison et al., 1990; 4. Arluison et al., 1994; 7. Code and Fallon, 1986; 10. Fendt et al., 1997; 11. Fort et al., 1994; 12. Geisler and Zahm, 2006; 13. Gray and Magnuson, 1987a; 14. Gray and Magnuson, 1987b; 15. Gray and Magnuson, 1992; 16. Gray, 1993; 17. Higgins and Schwaber, 1983; 18. Lechner and Valentino, 1999; 20. Moga and Gray, 1985; 22. Otake and Nakamura, 1995; 23. Panguluri et al., 2009; 26. Poulin et al., 2006; 27. Rao et al. 1987; 29. Rodaros et el., 2007; 30. Sakanaka et al., 1986; 31. Schwaber et al., 1988; 36. Vankova et al., 1992; 37. Veening et al. 1984.

Enkephalin (ENK)-containing fibers in the Ce were shown to originate from the extended amygdala (anterolateral BST, medial amygdaloid nucleus) and cortex-like amygdaloid nuclei (basomedial, ant cortical). In addition, the ventromedial hypothalamic nucleus and pontine parabrachial nucleus provide an ENKergic innervation to the Ce (Poulin et al., 2006). Hypothalamic afferents reaching the Ce contain a variety of other neuropeptides as well, e.g., the lateral hypothalamic area sends CRF and dynorphin (DYN)-expressing fibers to the Ce (Zardetto-Smith et al., 1988; Uryu et al., 1992). Lesioning studies indicate that vasoactive intestinal peptide (VIP) positive neurons in the supramammillary region and adjacent lateral hypothalamus project mainly to CeL (Baek et al., 1988). The Ce further receives afferents containing alpha-melanocyte stimulating hormone (a-MSH) from the arcuate nucleus (O'Donohue and Jacobowitz, 1980), non-hypophysiotropic thyrotropin-releasing hormone (TRH) from the parvocellular paraventricular hypothalamus (Wittmann et al., 2009), and hypocretin (HYP, also called orexin) from the perifornical nucleus and dorsal/lateral hypothalamic areas (Peyron et al., 1998). Tuberoinfundibular peptide of 39 residues (TIP39)-containing neurons localized in the subparafascicular thalamus and calcitonin gene-related peptide (CGRP)-expressing neurons in the posterior thalamic also innervate the Ce (Dobolyi et al., 2003; D'Hanis et al., 2007).

Among brainstem regions projecting to the Ce, the parabrachial nuclei provide peptidergic input with a high diversity. These afferents contain substance P (SP), NT, DYN, ENK and CGRP; however, cholecystokinin (CCK), somatostatin (SOM) and VIP were not detected in Ce-projecting parabrachial neurons (Shimada et al., 1985; Code and Fallon, 1986; Schwaber et al., 1988; Yamano et al., 1988a; 1988b; Block et al., 1989; Poulin et al., 2006; D'Hanis et al., 2007). The Ce is also innervated by SP- and leucine-ENK (LENK)-expressing afferents originating in the midbrain periaqueductal gray (Li et al., 1990). Moreover, the rat Ce receives CCK-containing dopaminergic fibers from the substantia nigra pars compacta/ventral tegmental area (Seroogy et al., 1989). Serotonergic projections from the dorsal raphe nuclei reaching the Ce further colocalize SP, LENK and CRF (Li et al., 1990; Uryu et al., 1992; Petrov et al., 1994), whereas projections from the laterodorsal tegmental nucleus contain SP and/or are cholinergic (Petrov et al., 1994). The Ce further receives neuropeptide Y (NPY)-, SOM-, NT-, MENK- and DYN-B-expressing afferents colocalizing tyrosine hydroxylase (TH), and a minor phenylethanolamine-N-methyl-transferase (PEMT)-containing projection from the nucleus of the solitary tract (Riche et al., 1990; Zardetto-Smith and Gray, 1990). In contrast, catecholaminergic projections from the ventrolateral medulla to the Ce colocalize NPY and PEMT (Zardetto-Smith and Gray, 1995). Fibers containing opiocortin (OPC) that reach the amygdala (corresponds probably to proopiomelanocortin, a precursor of adrenocorticotropin, beta-lipotropin, a-MSH and beta-endorphin (PubMed database, Gene: Pomc, Gene ID: 24664, updated on 5-Feb-2012)) were also reported to arise in the commissural nucleus tractus solitarius (Sim and Joseph, 1994).

4.2. Peptidergic Output

Projection neurons of the Ce send peptidergic fibers to most of the target regions, and these fibers innervate specific subregions in the BST, hypothalamus, thalamus and brainstem, which have been comprehensively studied in rodents (Table 2). CRF-positive Ce neurons important for regulating stress responses project to the BST, hypothalamus (lateral

hypothalamus, ventromedial nucleus), thalamus (paraventricular nucleus) and several brainstem areas, where they target the midbrain central gray, parabrachial region, caudal pontine reticular nucleus, ventral tegmental area, raphe nuclei, locus coeruleus, pericoerulear region and the nucleus of the solitary tract/dorsal vagal complex in rat (Veening et al., 1984; Sakanaka et al., 1986; Gray, 1993; Otake and Nakamura, 1995; Fendt et al., 1997; Van Bockstaele et al., 1998; Lechner and Valentino, 1999; Rodaros et al., 2007; Panguluri et al., 2009). The BST further receives LENKergic, NTergic and SPergic fibers from the Ce (Rao et al., 1987; Arluison et al., 1994), and the NTergic fibers probably coexpress CRF (Veinante et al., 1997), because CRF neurons of the Ce also target the BST (Sakanaka et al., 1986). SOM- and NT-containing Ce neurons also project to the midbrain central gray, parabrachial nuclei and dorsal vagal complex (Higgins and Schwaber, 1983; Veening et al., 1984; Moga and Gray, 1985; Gray and Magnuson, 1987b; 1992; Panguluri et al., 2009), but in addition to these projections, NT-positive Ce neurons innervate the lateral hypothalamus, substantia nigra pars lateralis and ventral tegmental area (Vankova et al., 1992; Allen and Cechetto, 1995; Geisler and Zahm, 2006).

Another peptide found in Ce neurons projecting to the midbrain central gray is galanin (GAL) (Gray and Magnuson, 1987a). Lesioning experiments of the stria terminalis further indicate that NPY neurons in the centromedial amygdala (including intraamygdaloid BST) may provide efferents to the BST, laterobasal septum and suprachiasmatic nucleus (Allen et al., 1984). Moreover, in rodents, a large proportion of LENK-, CRF-, NT-, NPY- and SP-positive afferents originating in the Ce were shown to innervate the lateral part of the BSTL (Allen et al., 1984; Sakanaka et al., 1986; Rao et al., 1987; Gray, 1993; Arluison et al., 1994).

5. TOPOGRAPHICAL ORGANIZATION OF EFFERENT PROJECTIONS

The afferent and efferent projections of the rodent amygdala and particularly those of the Ce are well studied (e.g., Krettek and Price, 1978; Ottersen and Ben-Ari, 1979; Ottersen, 1980; Ottersen, 1981; Cassell et al., 1986; Jolkkonen and Pitkänen, 1998) and reviewed by several authors (e.g., Cassell et al., 1999; Pitkänen, 2000; de Olmos et al., 2004; Knapska et al., 2007). Therefore, we focused on the comparison of the topographical arrangement of neurons projecting to defined subcortical regions and quantified the percentage of the projection neurons, which target four different brain areas mediating responses to emotionally relevant and/or loud acoustic stimuli (Figure 2). These four target areas that are involved in major aspects of acoustic arousal, the sensitization of the acoustic startle response and fear conditioning after presentation of acoustic stimuli are located within different regions of the brain; namely the SI in the forebrain, the NPV in the hypothalamus, the PNR in the pons and DMX/NST complex in the medulla (Figure 3). The results show that (i) each of these brain areas is targeted mostly by separate populations of neurons in the Ce, (ii) double-labeled neurons occur but are rare in every combination of injected targets, and (iii) the contribution of different subnuclei of the Ce appears to be different for each target studied.

Before going into a more detailed presentation and discussion of results, some methodological considerations have to be made. Projections of the Ce were studied using True Blue (TB) and Fluorogold (FG), both of which were proven to be reliable retrograde tracers (Kuypers et al., 1977; Bentivoglio et al., 1980; Schmued and Fallon, 1986; Pieribone

and Aston-Jones, 1988). TB was actively taken up by axon terminals and possibly also via damaged axons because we failed to inject TB iontophoretically, as proposed by Schmued and Heimer (1990). Since the injection with a Hamilton syringe always produced a rather large necrosis, it is possible that some damaged axons contributed to the observed projection patterns. In contrast to the mechanical injection method, FG was injected iontophoretically. This always produced circular injection sites with a small necrotic spot in the center of the injection site indicating the position of the electrode tip.

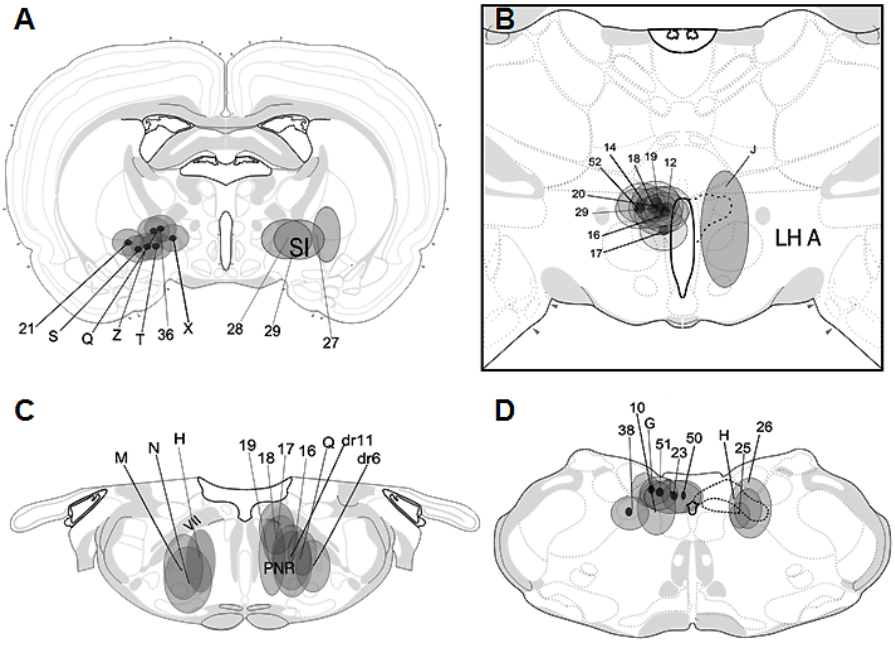
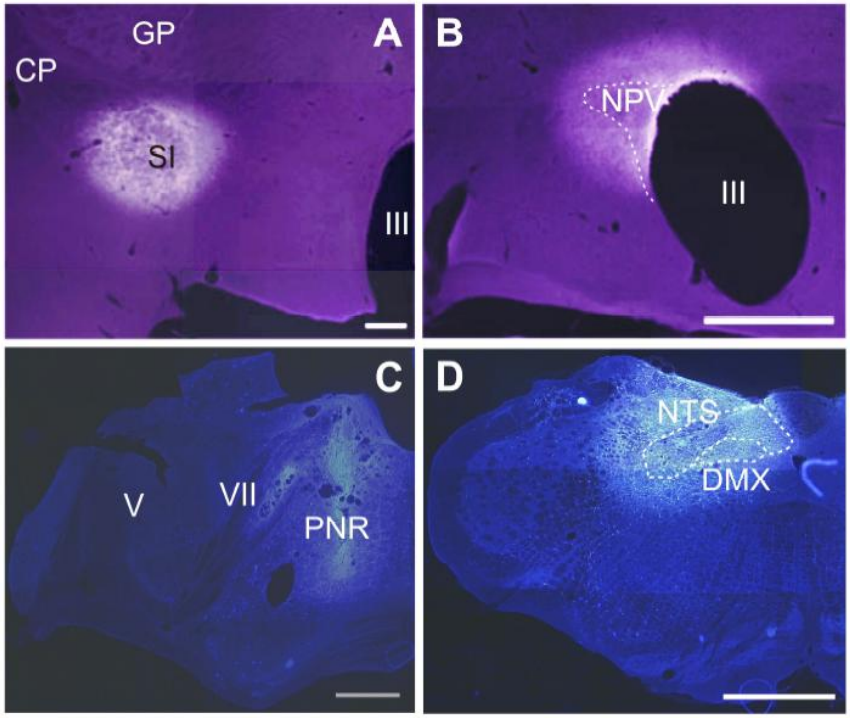


Figure 2. Tracer injections carried out in male Wistar rats (n=77, cases with misplaced injections discarded). Injection sites into the SI (A), the NPV (B), the PNR (C) and the DMX/NTS (D). Circled numbers indicate injections of representative cases presented in Figure 4. Injections on the right side are always Fluorogold (FG) injections. The dark spot shows the small necrosis resulting from iontophoretic injection, and the larger circle demarcates the circular diffusion zone of FG within the tissue. Three FG-injections (#H, M, N) in C are made with a Hamilton syringe. The injections with True Blue (TB) are always indicated on the left side. These injections were always made by means of a Hamilton syringe and are often larger. Here, the oval area demarcates the core of the injection. The figures show only those injections that are located within the borders of the respective brain areas. Injections that failed to hit the area or that were mostly outside of the area of interest were omitted. Fat dashed lines in B and D outline the NPV and the DMX/NTS, respectively. The figures were prepared using the software files of Brainmaps (Swanson, 1992) and the Open Source software Inkscape (version 0.45).

The size of the injection site, i.e., the extent of the diffusion of the dye, can be controlled by the current and the opening tip of the glass capillary during iontophoretic injections (Schmued and Heimer, 1990). We have also checked whether both tracers produce major differences in their staining patterns. For this reason, targets that were normally injected with FG (e.g., NPV) were also injected with TB. These “crossover injections” revealed equal staining patterns, although, in general, TB injections produced a higher number of stained neurons, probably due to the larger injection sites discussed above. A further drawback of TB

compared to FG is rapid bleaching of the former tracer. Despite the failure to inject TB iontophoretically, the advantages of the two tracers, especially when used together, outweigh their drawbacks. These advantages include the different emission wavelengths of TB and FG at the same excitation wavelength, allowing the simultaneous observation of both tracers, their comparable transport properties and their long-term stability within the cell body.



Abbreviations: see list. Calibration bars = 500µm (A), 300 µm (C), 1mm (B, D).

Figure 3. Example of injection sites into the four target areas. A: FG injection into the SI. In the center of the injection, a small necrotic spot is visible, indicating the location of the needle tip. Around this spot, the tracer diffuses concentrically. B: Injection into the NPV. Here, the concentric diffusion zone is interrupted by the third ventricle. C: TB injection into the PNR. Due to the use of a Hamilton syringe, the necrotic and diffusion zones are larger than after iontophoretic injections with FG. D: TB injection into the DMX/NTS area. The NTS and DMX are outlined by dashed lines.

A general problem in all tracer studies is the uptake of the tracer by intact fibers. This uptake by fibers of passage can produce false positive labeling. For TB, an uptake by intact fibers has been denied (Skagerberg et al., 1985), but other authors reported such uptake (Sawchenko and Swanson, 1981). For FG, uptake was shown mostly in damaged (Schmued and Fallon, 1986) but not intact axons (Pieribone and Aston-Jones, 1988; Kobbert et al., 2000), but also see Dado et al. (1990). Thus, a certain amount of false positive labeling is possible in TB injections. However, due to the similar pattern of retrogradely labeled neurons in the Ce after injections of both tracers, the main concern is the comparability of quantitative rather than qualitative results. Therefore, the percentage of retrogradely labeled neurons in individual subnuclei of the Ce, and not the absolute numbers of labeled neurons, will be provided (Table 3).

Table 3. Details of animals and injections

Case	Weight (g)	FG (iontophoretic injection)	TB (Hamilton syringe)	Electrode diameter (μm)	Duration (min.)	Survival time (days)
Wis J	290	SI	**NPV**	40	15	13
Wis S	340	**SI**	NPV	20	15	14
Wis T	300	**SI**	NPV	30	20	14
Wis Z	310	**SI**	NPV	45	20	10
Wis X	330	**SI***	PNR	40	20	10
dr11	320	SI	**PNR***	30	15	13
dr6	500	SI	**PNR***	25	10	14
Wis 36	270	**SI***	-	30	14	9
Wis Q	310	**SI***	**PNR***	40	20	14
Wis 21	300	**SI**	-	50	15	9
Wis 12	290	**NPV**	NTS/DMX	30	20	12
Wis 14	320	**NPV**	NTS/DMX	30	20	12
Wis 25	300	NPV	**NTS/DMX**	55	15	8
Wis 26	310	NPV	**NTS/DMX**	40	15	8
Wis 52	280	**NPV**	NTS/DMX	30	20	15
Wis 18	440	**NPV**	**PNR***	30	15	10
Wis 19	400	**NPV***	**PNR**	25	15	10
Wis 16	360	**NPV***	**PNR***	25	20	10
Wis 17	380	**NPV**	**PNR**	35	10	10
Wis 29	290	**NPV***	**SI***	50	15	8
Wis 27	300	NPV	**SI**	80	15	8
Wis 28	300	NPV	**SI***	40	15	8
Wis 20	300	**NPV***	-	40	15	9
Wis H	320	***PNR***	**NTS/DMX**	50	15	10
Wis M	300	***PNR***	NPV	40	15	11
Wis N	300	***PNR***	NPV	-	-	11
Wis 50	280	**NTS/DMX***	-	50	-	15
Wis G	300	**NTS/DMX***	PNR	35	15	10
Wis 51	280	**NTS/DMX***	PNR	20	20	15
Wis 38	270	**NTS/DMX**	-	25	-	10
Wis 23	270	**NTS/DMX**	-	30	15	9
Wis 10	290	***NTS/DMX***	NPV	-	-	10

Bold typesetting indicates the occurrence of the injection site in Figure 2. All injections using TB were made with a Hamilton syringe and most FG injections were made by means of iontophoresis. The injections of FG that were made with a Hamilton syringe were set in italics. Injections marked with an asterisk were quantified, and the data was presented in Figure 5.

5.1. Injections into Individual Regions

5.1.1. Injections into the Substantia Innominata (SI)

The substantia innominata is an ill-defined region between the olfactory tubercle, the anterior commissure, the septal area and the amygdala (Reil, 1809; Grove, 1988a; 1988b; Heimer et al., 1997). Cholinergic projections originating from magnocellular neurons in the substantia innominata have a significant modulatory influence on the cortex (Jones, 2004) and are critical for emotional arousal (rev. in Cahill, 2000), as indicated by two lines of evidence.

Stimulation of the amygdala induces an EEG activation in the cerebral cortex (low-voltage fast activity), which is mediated via the cholinergic projection from the SI and can be blocked by the muscarinic receptor antagonist scopolamine (Dringenberg and Vanderwolf, 1996).

Furthermore, lesions of the Ce in the amygdala or substantia innominata lead to severe deficits in attentional processing in experimental animals (Gallagher and Schoenbaum, 1999; Han et al., 1999). Considering the profound reciprocal connections between the CeM and SI (Grove, 1988a; 1988b), the CeM is indeed in a strong position to influence the SI. Since the CeM projections do not target the cholinergic projection neurons directly (Jolkkonen et al., 2002), fibers originating in the CeM might act on the cholinergic neurons via a GABAergic disinhibition.

Ten of our injections were successfully placed into the SI (Figure 2A, 3A). Figure 4 (SI) shows the distribution of retrogradely labeled neurons along the rostrocaudal axis of the Ce from a representative case (WIS X). Labeled neurons were found in all subnuclei of the Ce. The density of labeled neurons appears equal within the CeL and CeM, with no apparent clustering of neurons. Labeling in the ClC was mainly found in its rostral part. Quantification of retrogradely labeled neurons in the Ce (in five cases) revealed that only 60-80% of neurons are located in the CeM (Fig 5A). A rather high portion of neurons, corresponding to about 20-40% of all neurons projecting to the SI, was found in the CeL. Furthermore, retrogradely labeled neurons were seen regularly, but in a much smaller percentage (~5%), in the ClC.

In an extensive study on the afferents of the SI, Grove (1988b) reported a projection from the Ce to the dorsal SI. Although there is no differentiation between subnuclei of the Ce in this study, it can be seen in her Figure 4 that the majority of projection neurons are located in the CeM, whereas the lateral portions of the Ce are largely devoid of neurons.

In our study, we also observed the heavy projection from the CeM to the SI. However, a considerable proportion of projections to the SI arose in the CeL. A rather rough quantitative analysis revealed that a fraction of between 15-40% of neurons in the CeL contributes to this projection (cf. Figure 5A). These differences are explainable mainly by differences in the size or small variations in the localization of injection sites. Thus our findings are consistent with the observations of Petrovich and Swanson (1997), who also described an innervation of the SI (dorsal SI adjacent to the BST) after injecting the anterograde tracer PHAL into the CeL, and confirmed the CeL-SI projection using a retrograde tracing technique.

5.1.2. Injections into the Nucleus Paraventricularis Hypothalami (NPV)

The NPV is a small nucleus at the dorsal border of the third ventricle with two small wing-like lateral extensions. In the rat, the NPV is regarded as a tripartite cluster of magnocellular neurons that are embedded in a shell of parvocellular neurons that can be divided in up to five subnuclei (Swanson and Kuypers, 1980). Neurons in the magnocellular division of the NPV contain the peptides vasopressin and oxytocin, and they project to the posterior lobe of the pituitary gland (hypophyseal gland), where they secrete the two peptides into the general blood circulation (Armstrong, 1995). In contrast, the parvocellular division of the NPV projects to the neurohaemal zone of the hypothalamus, which is the origin of the portal circulation that transports releasing and inhibiting factors to the anterior pituitary gland to modulate its secretion. A direct projection of the parvocellular NPV to preganglionic sympathetic neurons in the thoracic spinal cord can regulate the function of the cardiovascular system and other autonomic systems directly, as well as indirectly, via the adrenal gland (Caverson et al., 1984; Armstrong, 1995; Motawei et al., 1999; Pyner and Coote, 1999).

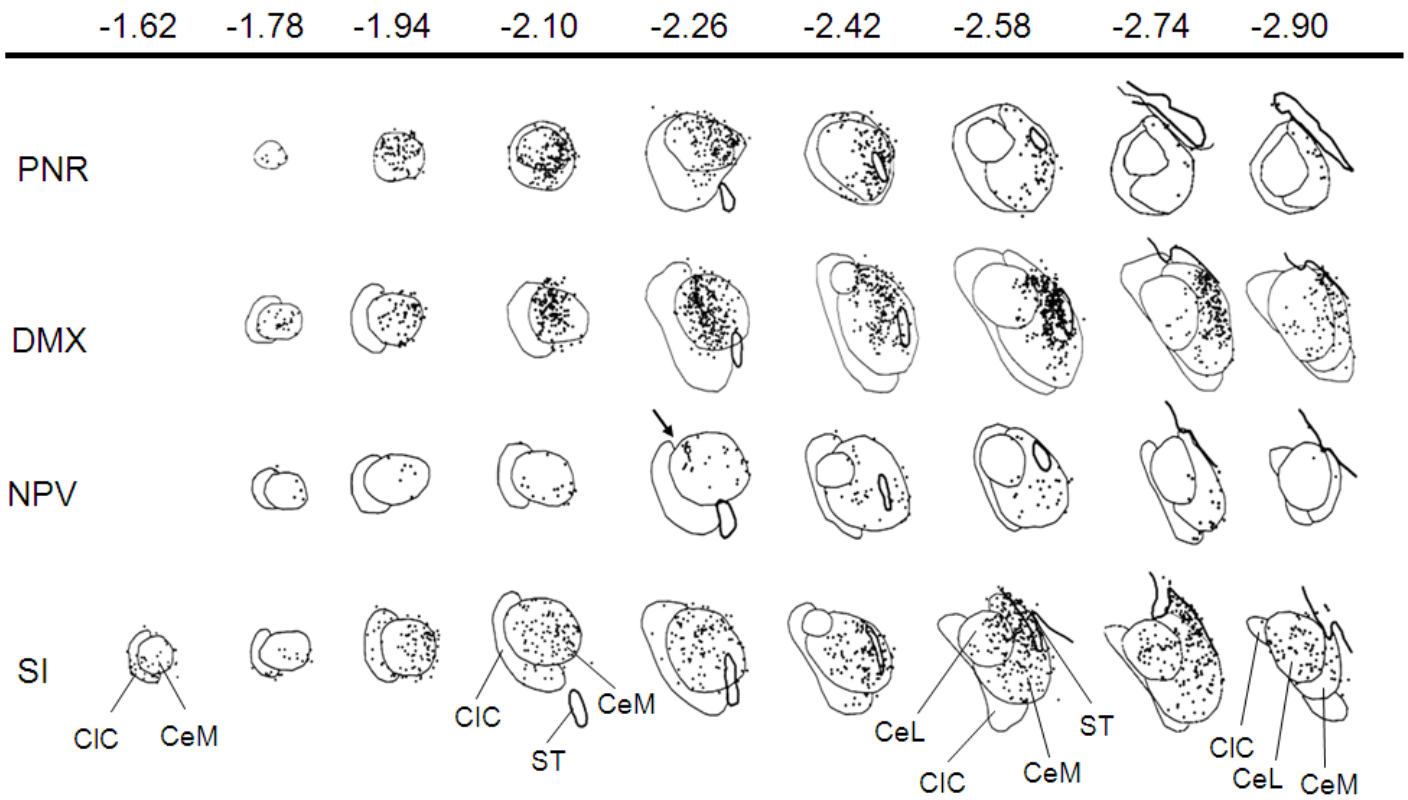


Figure 4. Comparison of the pattern of projection neurons along the rostrocaudal extent of the Ce after injections into four different targets. Each pattern comes from an individual case (SI injection case Wis X; NPV and PNR injection case Wis 16; DMX/NST injection case Wis 50). Every dot in the drawings represents a single neuron. The arrow in the third row (injection into NPV) points to a cluster of neurons regularly found at rostral levels. Numbers on top indicate the approximate location from Bregma. Sections were analyzed with a Neurolucida system (MicroBrightfield, Williston,VT) composed of a microscope equipped with fluorescence optics (Axioskop, Zeiss, Jena, Germany), CCD camera (IM-60300-01A, Optronics Engineering, Goleta, CA), PC and the neurolucida software (Ver.2.1; MicroBrightfield Europe, Magdeburg, Germany). For combining drawings of Nissl sections and retrogradely labeled neurons, the plots were converted with a custom-made program (Convaxx3, courtesy of Tan Bayraktar, Düsseldorf) and imported into Corel Draw (CorelCorporation, Ottawa, ON).

Altogether, 10 of our injections were placed successfully into the NPV (Figure 2B, 3B). Due to the relative midsagittal position of the NPV, the injections were made at an angle of 10° from the midline in order to spare the sagittal sinus. After injections into the NPV, only a small number of retrogradely labeled neurons were found in the Ce. A representative case (Wis 16) is shown in Figure 4. The majority of labeled neurons were seen in the CeM along the entire anterior-posterior axis of the Ce. Only a very few neurons were found in the CeL and ClC. All labeled neurons appeared to be evenly distributed within the CeM, although a small cluster of neurons was found regularly in the rostral part (Figure 4 arrow). This cluster was very small and detected in only two to three consecutive sections, corresponding to a maximum distance of 250-500 µm. Quantification of neurons (in four cases) showed that about 90% of all retrogradely labeled neurons are localized in the CeM (Figure 5B). A small but rather constant number of about 5% of neurons was found in the CeL close to the border of the CeM, which might be located within the central intermediate nucleus described previously (McDonald, 1997; Cassell et al., 1999). The few neurons in the ClC were always located close to the ventral border of the ClC and CeM.

A projection from the Ce to the NPV was first described by Gray et al. (1989). A more detailed report by Marcilhac and Siaud (1997) about these projections revealed retrogradely labeled CRF-positive neurons in the Ce, and about two to eight labeled neurons in a 20µm slice, although Moga and Saper (1994) mainly reported a CRF-containing projection from the BST (ventral lateral part) to the hypothalamic NPV. Another study, in which the anterograde tracer PHAL was injected mostly into the CeL (Prewitt and Herman, 1998), produced results that are similar to those of Moga and Saper (1994). Therefore, the authors suggested that the CeL-BSTL-NPV, rather than the light CeL-NPV pathway, is important for the activation of the HPA axis (Prewitt and Herman, 1998). However, our results confirm the findings of Marcilhac and Siaud (1997), who observed that the majority of the neurons were located in the CeM and a few neurons were also found in the CeL. Due to differences in section thickness (50 µm in our study), we counted a maximum of 30 neurons per section, which were also located mainly in the CeM. The CeL and ClC contained only a small fraction of neurons, ranging from 5-10% (cf. Figure 5B). We cannot completely exclude a diffusion of tracer beyond the borders of the NPV; however, because we systematically mapped the Ce, we discovered the small cluster of projection neurons in the midrostral CeM, which did not extend more than 200-500 µm in length. This small cell group that appears to be interposed between the CeL and CeM probably corresponds to the intermediate subdivision of the Ce (McDonald, 1988). Thus, in addition to the CeL-BSTL-NPV pathway proposed by Prewitt and Herman (1998), there seems to be a direct, though light, CeM-NPV projection.

5.1.3. Injections into the Pontine Reticular Nucleus (PNR)

The pontine reticular formation is located in the ventral brainstem and consists of a network of small and large neurons that is heavily intertwined with fiber bundles (Jones and Yang, 1985). It harbors domains that regulate the sleep-waking cycle, cortical arousal, somatic motor mechanisms and motor responses to nociceptive stimuli (Shammah-Lagnado et al., 1987). The entire network can be parcellated into a lateral or parvocellular division and a medial gigantocellular division.

Both divisions fulfill different functions: whereas the parvocellular part chiefly receives afferents and is regarded as a receptive area, the magnocellular part projects heavily to the

spinal cord, and is the origin of the reticulobulbar and reticulospinal tract responsible for movements, e.g., of the face, jaws, eyes, head and limbs (Tohyama et al., 1979; Blessing et al., 1981; Lingenhohl and Friauf, 1994). The PNR is innervated by a broad range of brain areas, including the cerebral cortex, nucleus basalis, central amygdaloid nucleus, hypothalamic districts, zona incerta and field H1 of Forel. Additional inputs arise in the superior colliculus; the accessory oculomotor and deep cerebellar nuclei; the anterior pretectal nucleus; and the cuneiform, trigeminal, parabrachial, cochlear and vestibular sensory cell groups. The PNR further receives afferents from the substantia nigra, central gray, mesencephalic and magnocellular pontomedullary reticular formation and the spinal cord. However, caudal sectors of the PNR seem to receive heavier input from spinal and cerebellar regions than oral sectors (Shammah-Lagnado et al., 1987; Kandler and Herbert, 1991; Lopez et al., 1999). The giant neurons in the caudal PNR have attracted particular attention, as they receive input from the auditory system and project to motor neurons in the spinal cord (Koch and Schnitzler, 1997). Since they have a short-latency to auditory inputs, high firing thresholds and broad frequency tuning, they can respond to sudden loud acoustic stimuli and induce a simultaneous twitch of somatic muscles, called the acoustic startle reflex (Lingenhohl and Friauf, 1994; Lee et al., 1996; Lopez et al., 1999). Thus, the caudal PNR is regarded as the sensory-motor interface for the acoustic startle reflex, which is under the direct control of the amygdala (Rosen et al., 1991; Fendt et al., 1997).

Nine injections were placed successfully into the PNR (Figure 2C, 3C). Figure 4 (PNR) shows the distribution of retrogradely labeled neurons in the Ce (case Wis 16). Retrogradely labeled neurons were found along the entire rostrocaudal axis of the Ce, but were almost completely restricted to the CeM. In the CeL and ClC (most caudal aspect), a few retrogradely labeled neurons were occasionally observed (Figures 4-5). The density of labeled neurons was rather low in contrast to injections into the SI, although it was somewhat higher in the rostral third of the CeM. Quantification of neurons (in six cases) confirmed that the CeL and ClC do not substantially contribute to this projection (Figure 5C). Within the borders of the PNR, where our injections were placed, several subnuclei were described that are involved in a variety of motor functions as diverse as vocalization (Jürgens, 2002), motor reaction to pain (Gauriau and Bernard, 2002) and the modulation of the acoustic startle response (Koch and Schnitzler, 1997).

The most detailed description of these projections demonstrates that they originate exclusively from the rostral and medial aspects of the Ce, whereas the caudal portions are devoid of labeled cells. Furthermore, the labeled neurons outlined the medial border of the CeL (Rosen et al., 1991).

In contrast to the descriptions of these authors, however, we also found a few labeled neurons in caudal portions of the CeM, although the majority of neurons were clearly located in the rostral and medial parts of the CeM. A second but rather minor deviation from the results of Rosen et al. (1991) is the occurrence of a few labeled cells in the CeL and ClC. Both differences may have been caused by the different tracers and/or injection methods used (Hamilton syringe with TB in present study vs. iontophoresis using FG in Rosen et al., 1991).

Altogether, the finding that the vast majority of projections to the PNR originate in the CeM is interesting, because CRF-containing neurons in the Ce are mostly concentrated in the CeL, although CRF application into the caudal PNR enhances the acoustic startle reflex (Fendt et al., 1997).

5.1.4. Injections into the Dorsal Vagal Nucleus (DMX) and the Nucleus of the Solitary Tract (NTS)

The DMX and NST are two neighboring elongated and small-caliber nuclei located in the dorsomedial brainstem, often summarized as the DMX/NST complex. The DMX, together with the ambiguous nucleus, contains preganglionic parasympathetic neurons, which are under the direct influence of the amygdala (Standish et al., 1995). Neurons of the DMX send off axons reaching the heart, lung and gastrointestinal tract via the vagus nerve. These different target areas are represented in separate motor columns within the DMX: the rostral part innervating the organs of the upper abdomen, the middle part the heart and the caudal part the esophagus and trachea. Furthermore, several subnuclei are described at least in humans (Huang et al., 1993) and in the pigeon (Katz and Karten, 1983). The NTS is a highly integrative nucleus that receives input from many visceral receptors and organs, such as the heart and vascular system (e.g., baroreceptors), respiratory system and gastrointestinal system. Comparable to the DMX, the NTS also has a topographic organization (Saper, 2004).

Eight injections were centered in the DMX/NST complex (Figure 2D, 3D). Figure 4 (DMX) shows the distribution of retrogradely labeled neurons in the Ce after such an injection (case Wis 50). Of all injection sites, the projection to the DMX/NST complex was the heaviest, although due to the elongated shape of both the NTS and DMX, only a small portion of the complex could be filled. Neurons projecting to the DMX/NTS complex were found in the entire rostrocaudal axis of the Ce, but the vast majority of the labeled neurons were located in the CeM. In contrast to the labeling in the Ce after injections into many target areas, the projection to the DMX/NTS complex produced a rather focal labeling in the CeM, i.e., labeled neurons were not evenly distributed in the frontal plane but clustered within the dorsal/dorsolateral CeM. The CeL contained very few labeled neurons. At rostral levels, these neurons were located along the medial border of the CeL, whereas caudally, neurons were distributed in the entire CeL. Quantification of neurons in the Ce (in four cases) provided evidence that the CeL and ClC contribute to a direct, though small, projection to the vagus complex, which is even smaller than the projection to the SI (Figure 5D).

The DMX and NTS were treated here as a unit because of their intimate apposition along their entire extension and their small sizes. Because we used single injections, only a fraction of the DMX/NTS complex was filled and, thus, only a small proportion of dorsal vagus-projecting Ce neurons were labeled, although this injection produced the highest number of labeled neurons in the Ce. The projections from the amygdala to the DMX are well known (Hopkins and Holstege, 1978; Kalia et al., 1979; Kalia and Sullivan, 1982; Schwaber et al., 1982; Higgins and Schwaber, 1983; Takeuchi et al., 1983; Danielsen et al., 1989). Morever, the projection neurons in the DMX/NTS complex are thought to be to be under GABAergic influence (Sun et al., 1994).

Higgins and Schwaber (1983) studied the CeL-DMX projection in greater detail and reported labeled neurons in the CeM and CeL, but unfortunately the Ce was not examined in its rostrocaudal extent. Our data confirm their results and show that the projections to the DMX/NTS complex arise along the whole rostrocaudal axis of the Ce. In addition, the projections originate from a cluster of neurons in the rostral and dorsolateral parts of the CeM, supporting the hypothesis that the dorsal (dorsolateral) and ventral (ventromedial) divisions of the CeM are anatomically and functionally different (rev. in Cassell et al., 1999).

In fact, the little double labeling of Ce neurons can be predicted from the complementary distribution pattern of neurons after injections into the PNR and DMX/NTS complex, because

PNR-projecting Ce neurons are mostly located at the medial edge of the CeM, whereas DMX/NTS-projecting Ce neurons spare this zone (Figure 4).

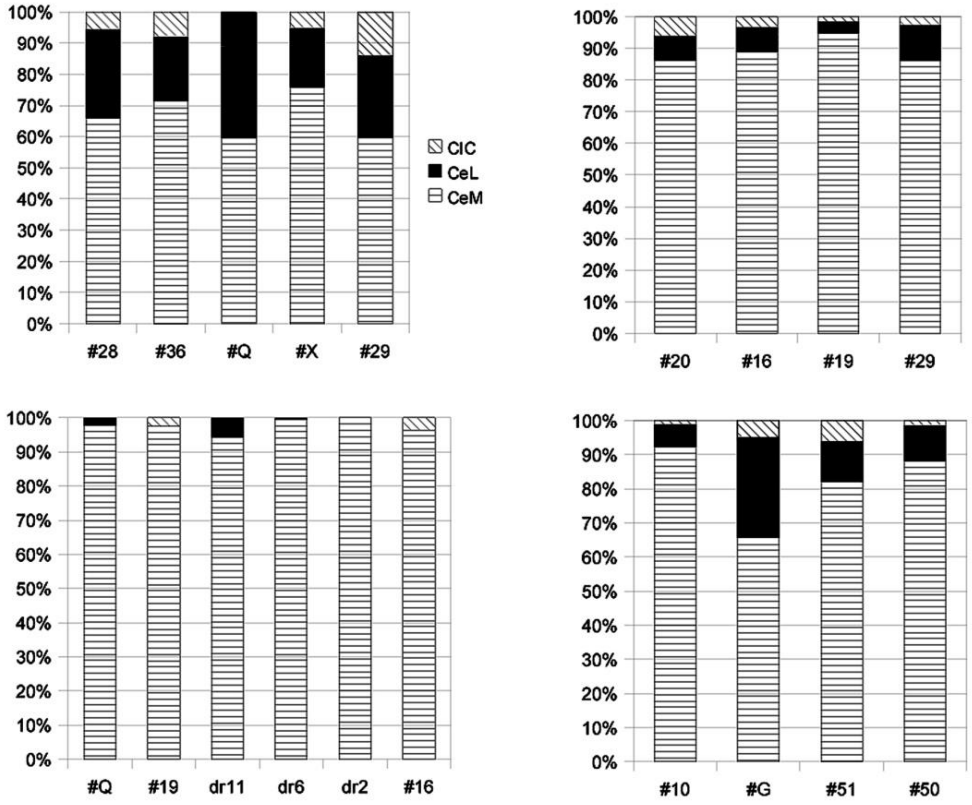


Figure 5. Percentage of retrogradely labeled neurons in the subnuclei of the Ce after injection of FG or TB into the SI (A), NPV (B), PNR (C) and DMX/NST (D). Each column represents a single case. Percentages were calculated by counting dots in each reconstruction; examples of these reconstructions are presented in Figure 4.

5.2. Quantitative Relations of Projection Neurons

Among all regions investigated, the NPV received the lightest and the PNR the heaviest input from the Ce. The majority of projection neurons were located in the CeM for all target areas investigated, whereas the other two divisions, the CeL and ClC, contributed to the projections to a variable extent. Many of the projection neurons found in the CeL and ClC were located at the borders of the CeM. A considerable fraction of projection neurons was located in the CeL, especially for the projections to the SI and DMX/NTS complex, whereas projections from the CeL to the NPV were very light and those to the PNR virtually absent. This is surprising, because we expected a rather high percentage of labeling in the CeL, mainly through the labeling of fibers damaged by injections via the Hamilton syringe. The very low or even absent labeling indicates that the PNR indeed does not receive a substantial projection from the CeL. These results demonstrate that the input from the Ce, at least to the

investigated brain areas, is heterogeneous. Thus, not only is the input to the Ce subnucleus-specific, but the output of the Ce also depends on the type of information that is conveyed. For example, the input from the brainstem to the CeL originates mainly from the lateral parabrachial nuclei (Bernard et al., 1993), whereas those to the CeM derive from the medial parabrachial nuclei (Bernard et al., 1993) and the posterior paralaminar thalamic nuclei (Linke et al., 2000; Wilhelmi et al., 2001).

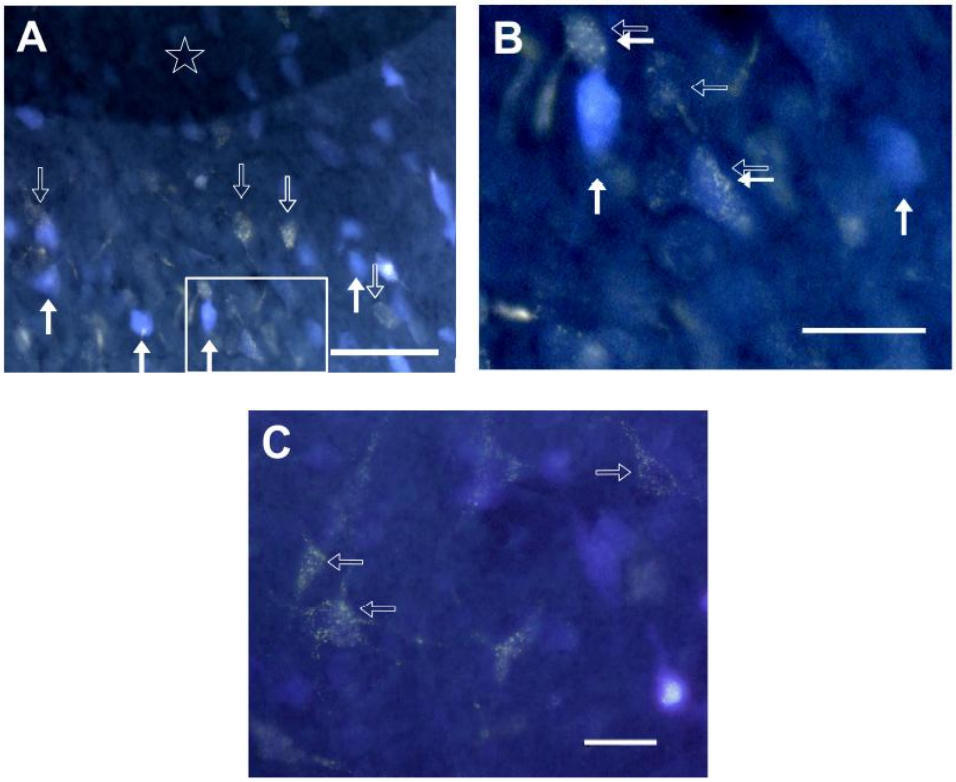


Figure 6. Examples of single- and double-labeled neurons. A: TB- and FG-labeled neurons in the CeM. TB-labeled neurons are homogeneously blue (solid arrows), whereas FG-labeled neurons often show a granular labeling (hollow arrows). The dark area at the top of the photograph (star) results from photo bleaching. The boxed area is enlarged in B. B: Two double-labeled neurons (pairs of hollow and solid arrows) and two single-labeled TB-labeled neurons (solid arrows) and one FG-labeled neuron (hollow arrow). C: Single-labeled TB- and FG-labeled neurons. Although the labeling with FG is weak, primary dendrites of these neurons are readily visible (hollow arrows). Calibration bars = 50 µm (A), 20 µm (B, C). Photographs were taken with a digital camera (Diagnostic Instruments Inc., Stirling Heights, MI, USA) on a Zeiss Axioplan 2 (Zeiss, Oberkochen, Germany). Photographs were arranged in Adobe Photoshop 7.0 (Adobe System) and adjusted for contrast and brightness.

5.3. Double-Labeled Neurons

One of the aims of this study was to analyze whether there are neurons that project to more than one target, as was successfully demonstrated for neurons of the basolateral complex of the amygdala, which has a cortex-like architecture (McDonald, 1991a; Pitkänen et al., 2000). Therefore, we also injected two tracers into some animals and analyzed retro-

gradely double-labeled neurons. At least one successful double injection for each combination of injections was available. In all these preparations, double-labeled neurons were regularly found in the Ce alongside single-labeled neurons, but these were rare, which is in agreement with previous observations (Thompson and Cassell, 1989; Fritz et al., 2005). In preparations in which injection sites are relatively close to each other, like the cases of SI and NPV, double-labeling was not higher. This further indicates that the injections were spaced widely enough that they did not contaminate each other. Figure 6 gives examples from those cases in which both injections were located in the center of their designated target. Double-labeled neurons were differentiated due to the different color (TB = blue vs. FG = yellow) and their differential staining properties: while TB stained the soma and primary dendrites homogeneously, FB staining often showed a granular appearance (Figure 6). Figure 7 (case WIS Q) is an example of double labeled neurons in a successful attempt to map them; it indicates that the few double labeled neurons do not show a preferential topographical localization after injection of retrograde tracers into the SI and PNR (case WIS Q). Nevertheless, we cannot exclude the possibility that rapid bleaching properties of TB compromised the mapping of the double-labeled neurons with the same precision as the single-labeled neurons, and might have led to an underestimation of double-labeled neurons.

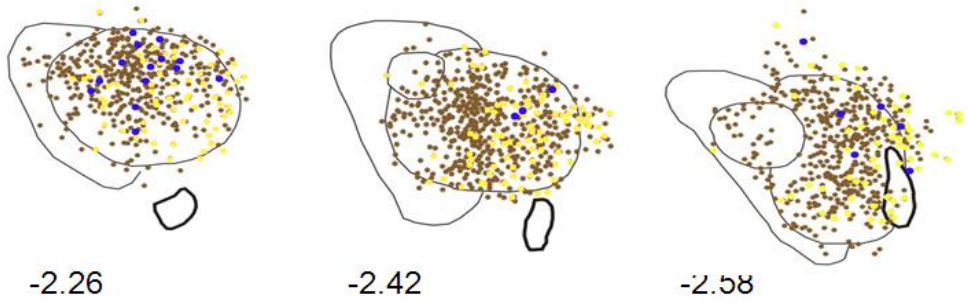


Figure7. Pattern of single- and double-labeled neurons after injection of retrograde tracers into the SI and PNR in case WIS Q. *Brown dots:* Injection of TB into PNR; *Yellow dots:* Injection of FG into SI; *Blue dots:* Double labeled neurons (according to Fritz, 2008).

6. FUNCTIONAL IMPLICATIONS

A concept generally accepted is that the amygdala is part of a serial system connected in a hierarchical order with brain areas mediating Pavlovian fear conditioning (rev. in Davis, 1992; Pitkänen et al., 1997; LeDoux, 2000). In this framework, the Ce has the role of a relais that samples information and distributes this information to the rest of the brain. This view is corroborated by the widespread connections of the Ce (rev. in Pitkänen, 2000; Knapska et al., 2007). However, recent behavioral (Balleine and Killcross, 2006; Knapska et al., 2006; Wilensky et al., 2006), physiological (rev. in Samson et al., 2005) and anatomical data (Cassell et al., 1999; Linke et al., 2000; Wilhelmi et al., 2001; Knapska et al., 2007) challenge this view and propose a more active role of the Ce in the processing of information.

As already demonstrated by LeDoux et al. (1988), the projections of the Ce to different targets mediate responses as diverse as freezing and arterial blood pressure. Moreover, the various response types regulated by the Ce can vary considerably in the timeline of their

activation, suggesting involvement of different neuronal populations and systems. For example, after presentation of an aversive stimulus in a fear-sensitization paradigm that is regarded as a form of rapid contextual fear-conditioning (rev. in Yilmazer-Hanke, 2008), freezing (data not shown) and the heart rate and arterial blood pressure show an immediate increase, whereas there is a delay in the increase of the acoustic startle response and body temperature measured in a startle apparatus and using telemetry electrodes (Figure 8). Although conditioned freezing and the arterial blood pressure response show a steep increase after presentation of conditioned stimuli, different pathways are required for both responses, i.e., the Ce-midbrain central gray pathway is required for conditioned freezing responses, and the Ce-lateral hypothalamus pathway for the conditioned arterial pressure response (LeDoux et al., 1988). For the fear-sensitized acoustic startle response, the Ce-caudal PNR pathway is required (Hitchcock et al., 1989; Rosen et al., 1991; Lee et al., 1996). The Ce-locus coeruleus-NPV and Ce-BST-NPV pathways seem to be important for the stress-mediated activation of the HPA axis (Beaulieu et al., 1987; Prewitt and Herman, 1998; Palkovits et al., 1999; Makino et al., 2002), and possibly also stress-related effects on the metabolism, e.g., increases in body temperature by ACTH-induced glucocorticoid release (Nakamura, 2011), because lesioning of the Ce reduces noradrenergic activity in response to stress within the anterior and lateral hypothalamic areas, the arcuate and paraventricular nuclei of the hypothalamus and the bed nucleus of the stria terminalis (Beaulieu et al., 1987).

How the CeL-CeM-mediodorsal thalamus pathway or involvement of the ClC/CeL-CeM in other striatal-pallidal-like circuits (Cassell et al., 1999) may influence these behavioral responses is currently unknown. What is known, however, is that the CeL can block conditioned freezing responses by inhibiting the output of CeM neurons (Ciocchi et al., 2010; Haubensak et al., 2010), which probably project to the midbrain central gray, as indicated by the findings of LeDoux et al. (1988), and that stress decreases dopaminergic activity in the Ce (Beaulieu et al., 1987). Since Paré and coworkers have demonstrated that the main and medial paracapsular intercalated nuclei differentially regulate the output of the CeM and CeL (Royer et al., 1999), these amygdaloid nuclei also may be important for regulating specific types of visceromotor and somatomotor outputs of the Ce. A further line of support for this view comes from anatomical tract tracing studies that demonstrate that the Ce receives considerable input from sensory and sensory-related areas, including spinal, brainstem, thalamic and cortical afferents (rev. in McDonald, 1998; Pitkänen, 2000).

Moreover, the subnuclei of the Ce receive differential input from various sensory modalities. For example, the CeM receives direct posterior thalamic afferents, which form synaptic contacts with neurons that project to the substantia innominata (Wilhelmi et al., 2001), but these thalamic afferents spare the CeL (Linke et al., 2000; D'Hanis et al., 2007). Also, the projections from the parabrachial nuclei display a strong topographical relationship to functional subsystems within the Ce. The lateral-most part of the parabrachial nuclei, which integrates input from ascending nociceptive pathways, projects to the ClC (Bernard et al., 1993), and the ClC (including the amygdalo-striatal area) also receives input from the somatosensory cortex SII (McDonald, 1998) and calcitonin gene-related peptide (CGRP)-containing neurons from the posterior thalamus (D'Hanis et al., 2007). The adjacent part of the lateral parabrachial nuclei is involved in the ascending visceral sensory pathway and projects onto the CeL (Bernard et al., 1993), and the CeL further receives input from gustatory and visceral portions of the insula (McDonald, 1998).

In contrast, the CeM is innervated by the medial parabrachial nuclei receiving input from the ascending gustatory pathway (Bernard et al., 1993), gustatory thalamus (Ottersen and Ben-Ari, 1979; Turner and Herkenham, 1991) and the gustatory and visceral portions of the insula (McDonald, 1998).

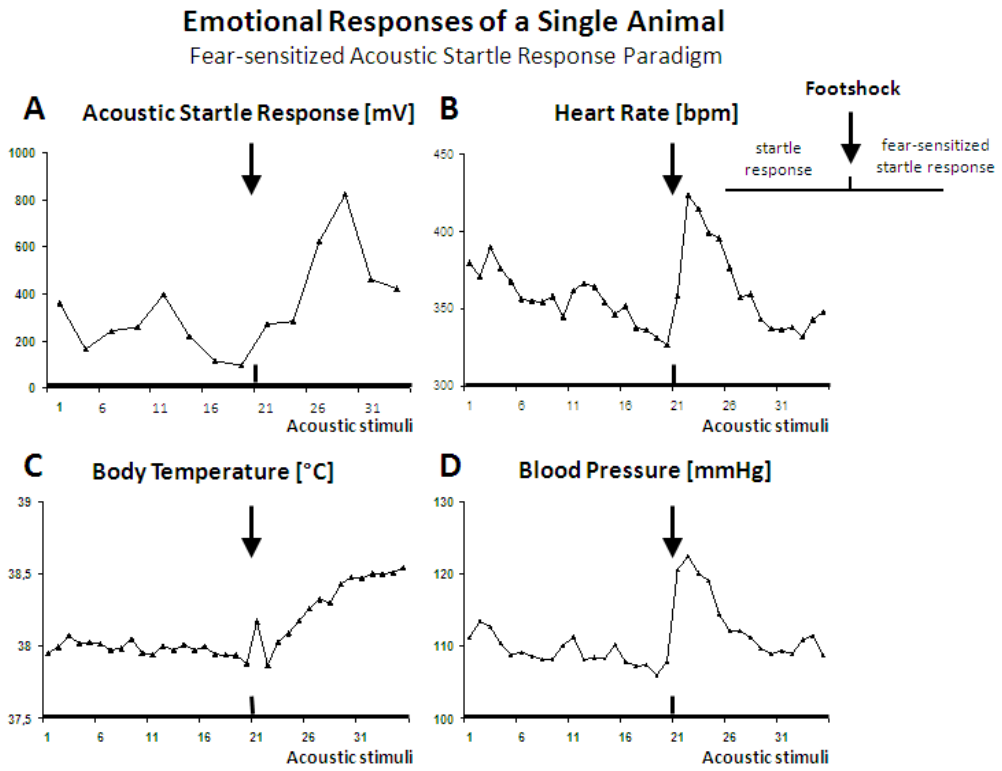


Figure 8. Time course of different types of emotional reactions in a single animal studied in the fear-sensitized acoustic startle paradigm before and after footshock in a startle chamber (for details: Yilmazer-Hanke et al., 2002). It is generally thought that all of these reactions are under the control of the Ce. A: The acoustic startle response was recorded using a piezoelectric platform during presentation of 10 kHz tones of 20 ms duration with rise/fall times of 0.4 ms at 105 dB SPL RMS superimposed on background noise at 55 dB SPL RMS (interstimulus intervals 30 s). B-D: The remaining parameters were measured using telemetry (TL11M2-C50-PCT electrodes, Data Sciences International, St. Paul, M 55126, USA) to monitor the blood pressure (sensor implanted into the abdominal aorta), electrocardiogram (ECG) and body temperature (latter two parameters measured with the aid of subcutaneous electrode leads).

Furthermore, the neurons in the CeL and CeM have different physiological properties ,suggesting that they are involved in different neuronal circuits (Martina et al., 1999), e.g., neurons in the ClC/CeL respond to noxious stimuli, whereas neurons in the CeM do not (Bernard and Besson, 1990; Neugebauer and Li, 2002). The functional importance of the latter pathway is supported by experiments that compared c-fos expression using the Ce in appetitive and aversive conditioning protocols. Appetitive conditioning led to a significantly higher density of c-fos labeled neurons in the CeM than aversive conditioning. The activation of c-fos labeling in the CeL was similar in both conditioning setups, which suggested that

novelty, rather than the mode of the stimulus, is important for this activation (Knapska et al., 2006).

Evidence for the different functions of the Ce subnuclei also comes from investigations on the distribution of peptidergic neurons within Ce, co-expression of various peptides and co-localization of peptides with GABA. For example, SOM-, ENK-, CRF- and NT-expressing neurons are mainly concentrated in the CeL, but these neurons are also found in the CeM, whereas SP immunoreactive neurons are mostly confined to the CeM. In addition, GAL-, CCK- and VIP-positive neurons were reported in the Ce after colchicine treatment (Cassell et al., 1986; Roberts, 1992; de Olmos et al., 2004). CRF and NT are coexpressed in Ce neurons, whereas these neurons do not contain MENK (Veinante et al., 1997). Likewise, the CeL contains a dense population of neurons that contain ENK, whereas the percentage of ENK-expressing GABAergic neurons is rather weak in the CeM compared to the CeL and ClC (Poulin et al., 2008).

The specific peptidergic population in the Ce neuronal populations do not only differ in their targets (Tables 1 and 2), but also in their developmental origin (Bupesh et al., 2011), suggesting that neuronal populations regulating different motor functions of the Ce are born in different developmental domains. Our data show that neurons projecting at the same time to different target regions occur but are rare. Instead, the downstream projections of the Ce arise in different subregions of the Ce, and even when the neurons intermingle, they still target different downstream regions. Taken together, these data support the idea that the Ce is far more than a simple output station of the amygdala, and that the subdivisions of the Ce have different roles in the regulation of the behavior of the organism.

CONCLUSION

The Ce is well-known as a motor output center of the amygdala that regulates endocrine, autonomic and somatomotor functions. However, a careful analysis of its afferents shows that the Ce also receives a considerable amount of sensory information. Modality-specific inputs from subcortical and cortical areas converging onto subnuclei of the Ce, and downstream projections originating from the Ce subnuclei, show a high level of topographical organization. The intrinsic connectivity of the Ce, the organization of some of its projections (e.g., to mediodorsal thalamus, midline thalamic nuclei, substantia nigra, subthalamic nucleus) and developmental data further suggest its involvement in striato-pallidal-like circuits. Furthermore, developmental, chemoarchitectural and connectional data indicate that there are separate sets of neurons in the Ce expressing different peptides and projecting to different target areas, which show little overlap. This is also consistent with functional differences observed, e.g., in the timeline and magnitude of various types of fear and stress responses like freezing, cardiovascular changes, rise in body temperature and the fear-sensitization of the startle response. Therefore, from a clinical point of view, it may be necessary to treat individual components of fear and stress responses separately to achieve the best success.

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