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CORTICO-THALAMIC PRINCIPLES DEFINE THE COMPLEXITY OF THE INTRA-AMYGDALAR CONNECTIVITY

THESIS BOOKLET

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Scientific Background and Aims

The way we perceive and interact with the world around us is highly dependent on our emotions. Virtually every situation in our lives elicits some emotion - if nothing else, boredom. In turn, the emotion elicited by an event or situation shapes how we react or behave. Some behaviors and actions have little significance in our lives, but sometimes they highly affect our well-being, mental and physical health, or our most important social relationships.

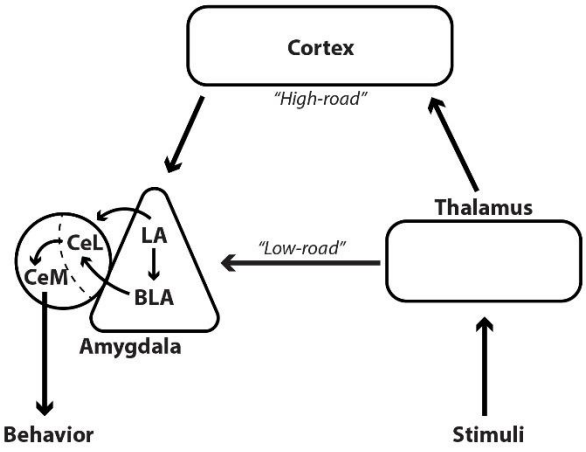
Considering their enormous significance, it is not surprising that the way emotions are formed and regulated in the brain has been extensively investigated in the previous century. Up to this point, psychologists, neuroscientists and physicians described the very foundations of the neurobiology of emotions, but there are still a multitude of unanswered questions in this regard.

In this doctoral thesis, I will briefly summarize our current knowledge on the neurobiology of emotion regulation, with special focus on fear-related processes, such as fear-learning, -expression, and -extinction. I will present a detailed anatomical and functional characterization of the neuronal networks involved in these processes, especially the amygdalar circuitry. Furthermore, I will incorporate the experimental data from our research group into this field of research with the intention of answering some open questions and resolving certain contradictions present in the current literature.

The regulation of fear learning, expression and extinction has been intensively investigated in the previous decades (LeDoux, 2014; Fanselow and Pennington, 2018; Zych and Gogolla, 2021). According to the currently accepted model, originally proposed by J. E. LeDoux (LeDoux, 2000, 2017), external and internal stimuli reach the amygdala through the thalamus and the cortex, where this information is integrated to form enduring memories (Figure 1). The amygdala, in turn, governs behavioral, autonomic and hormonal responses directly through its subcortical connections.

More specifically, at the level of the thalamus, the dorsal midline nuclei (DMT) and the nuclei surrounding the main auditory nucleus (medial geniculate nucleus – MGN) in the lateral thalamus (LT) are the two major sources of thalamic innervation to the amygdala. The model proposes that in a classical fear conditioning paradigm, during which a conditioned stimulus (CS; usually a tone) and an aversive unconditioned stimulus (US; usually a foot shock) become associated, ascending sensory signals are relayed through these thalamic nuclei directly to the

amygdala with short latency. However, careful investigation of the current literature raises questions about this proposition.



Modified from J. E. LeDoux

Figure 1. Classical model of amygdala-related fear conditioning. According to the model, CS and US signals reach the amygdala either directly through the thalamus ("low-road"), or indirectly through the cortex ("high-road"). In the amygdala, the lateral and basolateral nuclei receive thalamic and cortical axons converge providing the basis of signal association. Finally, axons from the lateral and basolateral nuclei drive neurons in the central amygdala, which innervates subcortical brain regions directly involved in motor, somatic and endocrine responses. Modified from J. E. LeDoux.

First, the DMT is mostly involved in the regulation of arousal levels, but not in primary sensory or nociceptive processing (Groenewegen and Berendse, 1994; Van der Werf et al., 2002; Vertes et al., 2015). However, since these nuclei are small, irregularly shaped and are surrounded by other, functionally diverse thalamic regions, specific investigation of the function of them has been challenging so far, so the exact function of these nuclei is still elusive.

Second, although the LT innervating the amygdala is generally considered as a relay station for auditory information, the actual nuclei innervating the amygdala (posterior intralaminar nucleus – PIL, suprageniculate nucleus – SG, medial MGN – mMGN, etc.) are rather multisensory in nature (LeDoux et al., 1985, 1987; Romanski and LeDoux, 1993; Zhang and Giesler, 2005). However, similarly to the DMT, due to their anatomical peculiarities, the specific investigation of the LT nuclei in question are also not straightforward. This notion on the other hand leads to the following questions. What kind of information can these multisensory nuclei transfer to the amygdala? And where is the actual site of CS/US association?

And third, although there are several contradictory results in the literature, it seems likely that these thalamic regions innervate different amygdala subnuclei with minimal overlap (LeDoux et al., 1990; Van der Werf et al., 2002). However, direct, systematic and quantitative comparison of the actual projection patterns of these thalamic regions in all amygdala subnuclei is still lacking.

Therefore, the main aim of the present thesis is to clarify the anatomy of the thalamic innervation of the amygdala (THESIS STATEMENTS 1-2). We sought to identify molecular markers that can reliably define the thalamic source of amygdala innervation and the borders of different amygdala subnuclei to achieve maximal anatomical precision.

At the level of the neocortex, the two most important regions known to provide significant projections to the amygdala are the medial prefrontal cortex (mPFC) (McDonald et al., 1999) and the higher-order associative regions of the temporal cortex (TeA) (Shi and Cassell, 1997). In accordance, these regions are strongly connected to the DMT and LT as well, respectively (Sesack et al., 1989; Shi and Cassell, 1997). According to the currently available data, the mPFC, especially its prelimbic (PrL) and infralimbic (IL) regions are actively involved in the precise tuning of the expression and extinction of fearful memories, respectively (Arruda-Carvalho and Clem, 2015). On the other hand, the temporal cortical regions contribute to the processing of complex sensory signals during fear conditioning and are involved in the long-term storage of fearful memories.

Although their involvement in fear-related processed are widely accepted, to the best of my knowledge, no studies compared their innervation patterns in the amygdala to each other and to thalamic innervation patterns directly. Therefore, the next major aim of this thesis was to describe and compare these cortical innervations in the different amygdala subnuclei (THESIS STATEMENT 3).

Within the mPFC, the most often cited subregions in the context of fear regulation are the prelimbic and infralimbic cortices. However, the anatomical definition of these regions is relatively vague (Le Merre et al., 2021). Moreover, some studies use other terms, such as ventral and dorsal mPFC, sometimes interchangeably for PrL and IL, respectively, sometimes including adjacent mPFC subregions, further complicating the interpretation of physiological and behavioral data. To resolve this issue, we sought to describe the distribution of amygdala projecting neurons in the mPFC, with special focus on the PrL and IL cortices (THESIS STATEMENT 4), based on the molecular profile we established in **Study 3**.

Circling back to the amygdala network model, once the information from these thalamic and cortical sources reaches the amygdala, the intra-amygdalar connections take over its processing. It is generally accepted that a relatively linear flow of information takes place in this structure, from the basolateral amygdala complex, and especially its lateral division (LA), towards the central amygdala (CeA) (Orsini and Maren, 2012).

However, there are several inconsistencies and unanswered questions regarding the intra-amygdalar connectivity. Most notably, although a straight lateral-to-central amygdala pathway is considered as a crucial element of this network, direct anatomical evidence for the existence of this pathway is lacking (Tovote et al., 2015). Therefore, the last aim of this thesis was to reliably map intra-amygdalar connections and compare them to the previously established thalamic and cortical innervation patterns (THESIS STATEMENT 5).

Thesis statements

Thesis statement 1: The amygdala is innervated by two prominent thalamic sources in a non-overlapping manner

We demonstrated that the vast majority (>90%) of amygdala-projecting thalamic neurons express calretinin (Calr) and are localized in the DMT and LT in **Study 1** and **Study 2**. In these studies, we used a combination of classical retrograde, Calr-dependent anterograde viral tracing techniques and multiple fluorescent immunohistochemistry to confirm these connections in both directions. However, these two studies investigated DMT-amygdala and LT-amygdala connections separately.

Therefore, we simultaneously labelled these two thalamic Calr-expressing populations using the same viral tools and directly compared their axon-densities in the amygdala. We used various molecular markers to identify and distinguish different amygdalar subnuclei in a biologically reliable manner. During these experiments we applied a custom-made ImageJ script specifically designed to tract and measure axon densities in high magnification fluorescent confocal images. According to these **unpublished results**, DMT and LT Calr-expressing cells innervate the major amygdala subnuclei in a quantitatively non-overlapping manner indeed. Namely, the DMT most heavily innervates the anterior basolateral nucleus (BLA) and the centrolateral nucleus (CeL), while LT sends strong projections to the LA, the amygdalostriatal transition zone (Astr) and the anterior basomedial nucleus (BMA).

These results indicate that ascending excitatory information of different nature from the thalamus may not directly converge in the major amygdala subnuclei. Specifically, these anatomical results suggest that arousal-related and sensory information from the thalamus (DMT, LT, respectively) mostly reach different amygdala regions.

Related publications:

- **Study 1: A highly collateralized thalamic cell type with arousal-predicting activity serves as a key hub for graded state transitions in the forebrain (2018),** Mátyás, F*., Komlósi, G.*, Babiczky, Á., Kocsis, K., Barthó, P., Barsy, B., Dávid, C., Kanti, V., Porrero, C., Magyar, A., Szűcs, I., Clasca, F., & Acsády, L., Nature neuroscience, 21(11), 1551–1562.

- **Study 2: Associative and plastic thalamic signaling to the lateral amygdala controls fear behavior (2020),** Barsy, B.*, Kocsis, K.*, Magyar, A., Babiczky, Á., Szabó, M., Veres, J. M., Hillier, D., Ulbert, I., Yizhar, O., & Mátyás, F., Nature neuroscience, 23(5), 625–637.
- **Unpublished resulst**

Thesis statement 2: The intercalated cells receive converging thalamic innervation

In THESIS STATEMENT 1, I concluded that the two thalamic sources give rise to a mostly non-overlapping innervation pattern in the amygdala. One prominent exception could be the dorsomedial intercalated cluster (dm-ITC) and the so-called supra-intercalated cluster of neurons (SIC) first described in **Study 2**. These γ-aminobutyric acid (GABA)ergic cells clusters play an important role in intra-amygdalar inhibition (see *Introduction*), although their exact functional contribution to fear-regulation is not completely elucidated yet.

In **Study 2**, we demonstrated that the SIC is selectively activated to the simultaneous presentation of CS and US, or US alone. Similar activation patterns were also reported for the dm-ITC cluster in previous studies (see *Introduction*). Furthermore, it was also shown that these regions receive LT innervation conveying noxious and sensory signals. However, in this study we did not quantify the strength of LT innervation. Furthermore, to the best of my knowledge, quantitative data for DMT projections in these clusters is also not available.

Therefore, parallel to the quantification of thalamic innervation of the major amygdala subnuclei described in THESIS STATEMENT 1, we also measured axon densities in the SIC and dmITC. According to our **unpublished results**, these clusters receive similarly strong innervation from both the DMT and LT. In fact, the density of labelled axons was comparable to the most strongly innervated amygdala subnuclei for both thalamic regions.

Taken together, our results indicate that thalamic innervation not only drives prominent excitatory pathways in the amygdala, but also shapes intra-amygdalar inhibition. Furthermore, we also demonstrated that GABAergic neuron clusters in question (SIC, dm-ITC) receive converging thalamic input from the DMT and LT, although the functional consequence of this concurrence is not clear yet.

Related publications:

- **Study 2: Associative and plastic thalamic signaling to the lateral amygdala controls fear behavior (2020),** Barsy, B.*, Kocsis, K.*, Magyar, A., Babiczky, Á., Szabó, M., Veres, J. M., Hillier, D., Ulbert, I., Yizhar, O., & Mátyás, F., Nature neuroscience, 23(5), 625–637.
- **Unpublished resulst**

Thesis statement 3: Cortical innervation of the amygdala resembles thalamic innervation patterns

In **Study 1**, **Study 2,** and **Study 3** connections between the DMT, LT and the neocortex were characterized. According to **Study 1** and **Study 3**, DMT is reciprocally connected to the mPFC, especially to its PrL and IL regions. On the other hand, **Study 2** demonstrated that the strongest neocortical partner of LT is the TeA region. We have not found evidence for significant direct connections between the DMT and TeA, nor between LT and mPFC in either direction.

Besides these thalamo-cortical and cortico-thalamic connections, the amygdala also receives neocortical afferents from the mPFC and TeA. In **Study 2** we demonstrated that LA is heavily innervated by the TeA, while data published in **Study 3** indicate that mPFC IT neurons target the amygdala, especially the BLA subregion and the dm-ITC. However, amygdala subnuclei-specific systematic comparison of mPFC and TeA afferents is lacking.

So, we virally labelled mPFC and TeA neurons and compared their projection patterns in the amygdala. Our **unpublished results** revealed that these two neocortical sources target different amygdala subnuclei indeed. Specifically, mPFC strongly innervates the BLA, the posterior basomedial nucleus (BMP), the centromedial nucleus (CeM) and, to some extent, Astr, while the most prominent targets of the TeA are the LA and Astr. Importantly, strongest mPFC and TeA innervation was observed in the nuclei that was also heavily innervated by DMT or LT, namely BLA and LA, respectively. Interestingly, the SIC received innervation from both cortical regions, the dm-ITC received most of its cortical input from the mPFC only.

Taken together, our results demonstrated that besides the non-overlapping thalamic innervation, cortical afferents from the mPFC and TeA are also separated in the different amygdala subnuclei. Furthermore, this separation resembles thalamic patterns, especially in the cortical regions of the amygdala (LA, BLA). These observations indicate that higher-order cortical information from different sources does not converge directly in these amygdala regions similarly to thalamic innervation. One notable exception could be the SIC, where thalamic axons also overlap extensively.

Related publications:

- **Study 1: A highly collateralized thalamic cell type with arousal-predicting activity serves as a key hub for graded state transitions in the forebrain** (2018), Mátyás, F*., Komlósi, G.*, Babiczky, Á., Kocsis, K., Barthó, P., Barsy, B., Dávid, C., Kanti, V., Porrero, C., Magyar, A., Szűcs, I., Clasca, F., & Acsády, L., Nature neuroscience, 21(11), 1551–1562.
- **Study 2: Associative and plastic thalamic signaling to the lateral amygdala controls fear behavior** (2020), Barsy, B.*, Kocsis, K.*, Magyar, A., Babiczky, Á., Szabó, M., Veres, J. M., Hillier, D., Ulbert, I., Yizhar, O., & Mátyás, F., Nature neuroscience, 23(5), 625–637.
- **Study 3: Molecular characteristics and laminar distribution of prefrontal neurons projecting to the mesolimbic system** (2022), Babiczky, Á., & Mátyás, F., eLife, 11, e78813.
- **Unpublished results**

Thesis statement 4: Amygdala subnuclei receive different innervation from mPFC subregions and layers

Although previous studies investigated subregional differences in mPFC innervation in the amygdala, common nomenclatural inconsistencies and the lack of a clear and routinely used definition for mPFC subregions make the interpretation of these studies complicated. While some studies divide mPFC to a ventral (vmPFC) and a dorsal (dmPFC) region, others use differentiate between PrL, IL and cingulate (Cg) regions, among others, but the exact correspondence between these divisions remains elusive (see *Introduction*). For example, does vmPFC include exclusively the IL, or the ventral part of the PrL is also a part of it? If the latter is true, where is the border between a dorsal and ventral PrL? Besides these open questions, to the best of my knowledge, the laminar origin of mPFC-to-amygdala innervation has not been extensively investigated subnuclei to subnuclei.

Therefore, we applied the molecular markers we defined in **Study 3** (calbindin, parvalbumin, Ctip2, FoxP2) to discover the subregional and laminar distribution of BLA-, BMP-, CeM- and Astr-projecting mPFC neurons labelled with classical retrograde tracing. According to these **unpublished results**, the vast majority of amygdala-projecting neurons were found in the PrL, IL, Cg and medial orbital (MO) regions. However, while BLA-projecting neurons were mostly found dorsally in the Cg and PrL subregions, most BMP-, CeM- and Astr-projecting cells were localized ventrally in the PrL, IL and MO, although some overlap was detectably, especially in the PrL and IL. Furthermore, while neurons innervating the basolateral nuclei (BLA, BMP) were found both in the layer 2/3 and 5, cells projecting to the striatal nuclei of the amygdala (CeM, Astr) were almost exclusively in the layer 5.

In sum, we confirmed earlier results indicating the subregional separation of mPFC innervation within the amygdala with previously unprecedented anatomical precision. Our results suggest that although different mPFC subregions indeed tend to innervate the amygdala differently, this separation is rather transitional than clear-cut. However, the basolateral complex and striatal amygdala receive innervation from different mPFC layers (L2/3-5 and L5, respectively), a notion that has not been published yet.

Related publications:

- **Study 3: Molecular characteristics and laminar distribution of prefrontal neurons projecting to the mesolimbic system (2022),** Babiczky, Á., & Mátyás, F., eLife, 11, e78813.
- **Unpublished results**

Thesis statement 5: There are several parallel pathways within the amygdala

Canonical models of amygdala-related associative learning propose a serial information flow from the LA to the CeA either directly or through the basal nuclei (see *Introduction*). However, thalamo-amygdalar and cortico-amygdalar innervation patterns described in THESIS STATEMENT 1-4 suggest the existence of several separated intra-amygdalar pathways. In **Study 2**, we proposed several possible intra-amygdalar pathways involved in fear conditioning and retrieval, although we did not investigate intra-amygdalar connections systematically in this study.

To clarify intra-amygdalar connectivity in a quantitative manner, we mapped direct connections between each amygdala subnuclei in a line of classical retrograde tracing experiments with special focus on LA and BLA. The **unpublished results** of these investigations revealed that 1) there is no strong direct connection between LA and BLA, 2) nor between each of these regions and the CeL. Furthermore, 3) LA innervation is most prominent to Astr and BMP, while 4) BLA does not provide strong innervation to any other amygdala subnuclei.

Taken together, we described a complex intra-amygdalar network in contrast to the classical, rather linear information flow model. Considering THESIS STATEMENT 1 and THESIS STATEMENT 3, a new model (Figure 10) can be proposed in which different thalamic (DMT, LT) and cortical (mPFC, TeA) innervations drive parallel excitatory pathways within the amygdalar network, most notably via the BLA and LA, respectively.

Related publications:

- **Study 2: Associative and plastic thalamic signaling to the lateral amygdala controls fear behavior (2020),** Barsy, B.*, Kocsis, K.*, Magyar, A., Babiczky, Á., Szabó, M., Veres, J. M., Hillier, D., Ulbert, I., Yizhar, O., & Mátyás, F., Nature neuroscience, 23(5), 625–637.
- **Unpublished results**

Conclusion

The present doctoral thesis focused on the brain network that regulates emotional behavior, especially fear learning. Although the research on the neurobiology of fear produced an enormous amount of data in the subsequent decades, it is still far from complete. Nevertheless, I tried my best to summarize the most important findings published so far, and I also tried to incorporate our own experimental data to the existing literature.

In brief, the amygdala has been identified as the central element of the network that controls fear learning and expression, through its afferent and efferent connections with the most important "limbic" structures of the brain. These include, but not limited to the DMT and LT, as well as the mPFC and the TeA. Although all of these structures underwent extensive investigation, certain details remained unresolved about their connections and functions. My research mainly focused on the anatomical characteristics of this network, with special focus on their molecular identity and the still abundant contradictions still present in the literature.

The most important findings summarized in this thesis were the following. (1) Different amygdala subnuclei receive innervation from Calr-expressing neurons of the DMT and LT in a mostly non-overlapping manner. (2) One point of convergence of thalamic innervation is the ITCs, specifically the SIC and the dmITC. (3) mPFC and TeA innervation is also mostly non-overlapping in the amygdala and this pattern resembles thalamic axon-arborization, especially in the LA and BLA. (4) mPFC innervation in different amygdala subnuclei (BLA, BMP, CeM, Astr) originates in different mPFC regions and layers. (5) Within the amygdala, the LA is a major source of innervation to other subnuclei, while the BLA seems to be connected to extra-amygdalar targets, contradicting previous assumptions to some extent.

Collectively, our results implicate the existence of at least two parallel thalamo-cortico-amygdalar loops involved in different aspects of fearful behavior (Figure 2). Anatomically these pathways are positioned to drive different amygdalar networks that, in turn, can modulate the activity of brain regions involved different aspects of behavior. However, we also identified the ITCs as important candidates for signal convergence in this network. In the future, electrophysiological and behavioral experiments could reveal the functional role of this apparent pathway separation, hopefully contributing to a better understanding of amygdala-related processes in the brain.

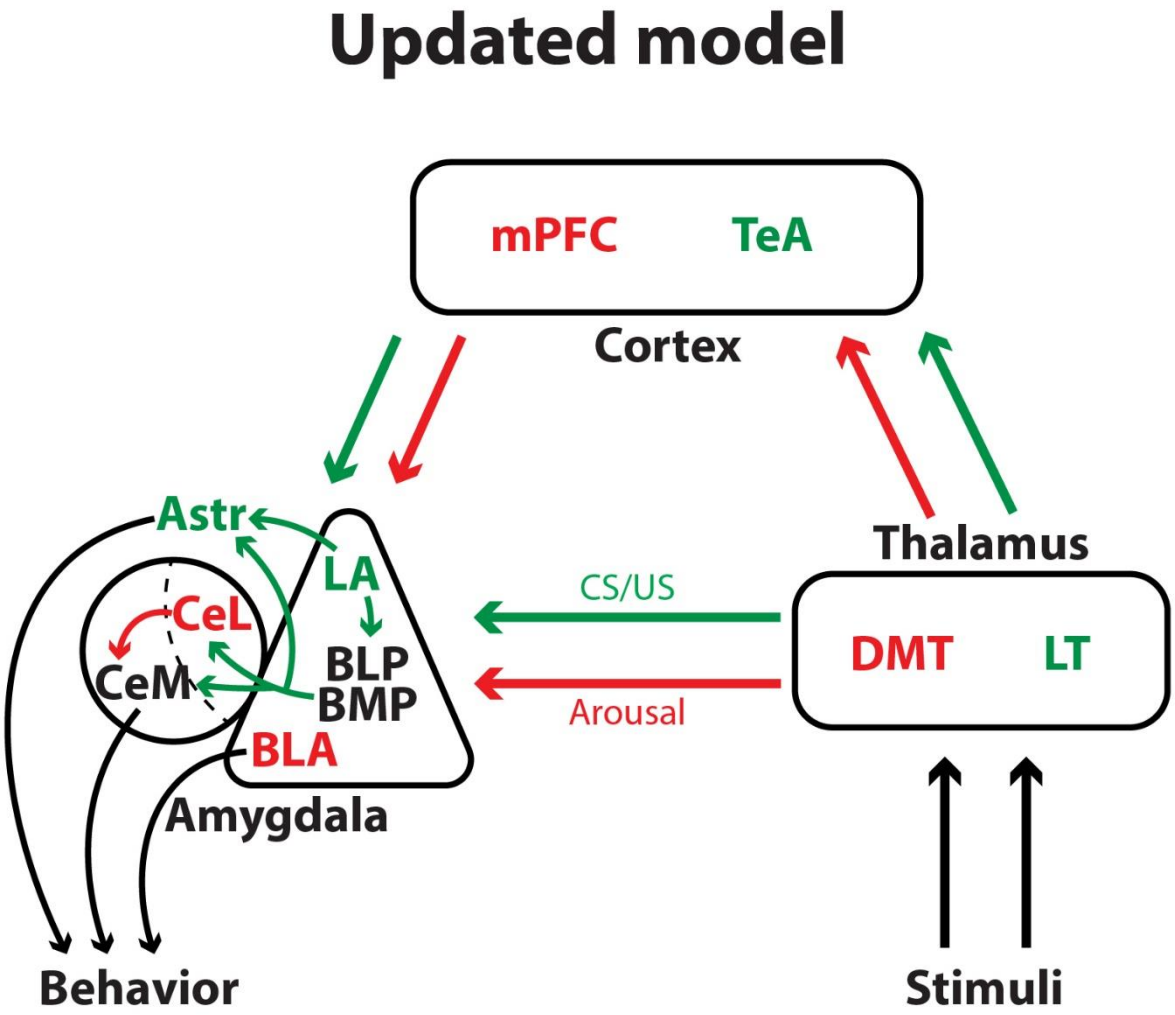


Figure 2. Proposed new model of amygdala-related fear conditioning. Different stimuli reach the amygdala via two separated thalamo-cortical pathways. We suggest that external stimuli (CS/US) are carried through the LT-TeA (green) pathway, while internal stimuli (arousal) through the DMT-mPFC (red) pathway. These pathways terminate in different amygdala subnuclei, most notably in the LA and BLA, respectively. In turn, LA drives a complex intra-amygdalar network, while BLA innervates extra-amygdalar targets involved in various aspects of emotional behavior. Astr – amygdalostriatal transition zone; BLA, BLP – basolateral amygdaloid nucleus, anterior, posterior part; BMP – basomedial amygdaloid nucleus, posterior part; CeL – centrolateral amygdaloid nucleus; CeM – centromedial amygdaloid nucleus; DMT – dorsal midline thalamus; LA – lateral amygdaloid nucleus; LT – lateral thalamus; mPFC – medial prefrontal cortex; TeA – temporal association area.

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