Afferent connections of the thalamic nucleus reuniens in the mouse

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Abstract

The nucleus reuniens (RE) is part of the midline thalamus and one of the major sources of thalamic inputs to the hippocampal formation and the medial prefrontal cortex. However, it not only sends strong efferents to these areas but is also heavily innervated by both brain regions. Based on its connectivity and supported by functional studies the RE has been suggested to represent a major hub in reciprocal hippocampal–prefrontal communication. Indeed, inactivation studies have demonstrated that this nucleus is particularly important for cognitive behaviors which depend on prefrontal–hippocampal communication, such as working memory or memory consolidation. However, besides its central role in mediating hippocampal–prefrontal communication, the RE is target of a multitude of other cortical and subcortical afferents, which likely modulate its function.

So far, however, studies that have systematically investigated the afferents of the RE have only been performed in rats. Because of the unique role of the mouse as a genetically accessible model system for mammalian brain circuit analysis we have mapped the afferent connectivity of the mouse RE using retrograde Fluoro-Gold tracing. Comparison with similar data from rats indicated a very high level of similarity in prefrontal and hippocampal afferents but some differences in afferent connectivity with other brain regions. In particular, our results suggest interspecies differences regarding the integration of the RE in circuits of fear, aversion, and defense.

KEYWORDS

amygdala, hippocampus, mouse, nucleus reuniens, prefrontal cortex, RRID: MGI:2670020, RRID:SCR_013672, RRID:SCR_014199, RRID:SCR_016844, subiculum

Abbreviations: 2Cb, second cerebellar lobule; Acb, nucleus accumbens; AI, agranular insular cortex; AM, thalamic anteromedial nucleus; BLA, basolateral amygdaloid nucleus, anterior part; BMP, basomedial amygdaloid nucleus, posterior part; CeC, central amygdaloid nucleus, capsular part; Cg1, Cg2, cingulate cortex (area 1, area 2); Ci, claustrum; CM, centromedial thalamic nucleus; DEn, dorsal endopiriform nucleus; DG, dentate gyrus; DLPAG, dorsolateral periaqueductal gray; DM, dorsomedial hypothalamic nucleus; DP, dorsal peduncular cortex; DTT, dorsal tenia tecta; Ect, ectorhinal cortex; Ent (Lent, Ment), entorhinal cortex (lateral, medial part); Hb, habenular nucleus; HC, hippocampus; IL, infralimbic cortex; InG, intermediate gray; LDTg, laterodorsal tegmental nucleus; LH, lateral hypothalamus; LPAG, lateral periaqueductal gray; LSD, LSI, LSV, lateral septal nucleus (dorsal, intermediate, ventral part); M1, M2, primary, secondary motor cortex; ML, MM, medial mammillary nucleus (lateral, medial part); MN, mammillary nuclei (ML, MM, and SuM); MO, LO, VO, orbital cortex (medial, lateral, ventral); MPA, medial preoptic area; mPFC, medial prefrontal cortex; MS, medial septal nucleus; PAG, periaqueductal gray; PaS, parasubiculum; PH, posterior hypothalamic area; Pir, piniform cortex; PrV, pontine reticular nucleus, ventral part; PRh, perihinal cortex; PrL, prelimbic cortex; PrS, presubiculum; PT, paratenial thalamic nucleus; PV, paraventricular thalamic nucleus; anterior part; RE, thalamic nucleus reuniens; Rh, rhomboid thalamic nucleus; RS/Cg, fused cingulate/retrosplenial area; RSA, anterior agranular retrosplenial cortex; RSG, anterior granular retrosplenial cortex; S, subiculum; S1 (S1HL), primary somatosensory cortex (hind limb region); SC, superior colliculus; SFI, septofimbrial nucleus; SN, substantia nigra; SuM, supramammillary nucleus; V1, primary visual cortex; VM, ventromedial thalamic nucleus; VTA, ventral tegmental area; ZI, ZIV, zona incerta (ventral part).

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1 | INTRODUCTION

The midline nuclei of the thalamus comprise the paraventricular and paratenial nuclei, as well as the rhomboid and reunions (RE) nuclei. Unlike the “specific” or “relay” nuclei of the thalamus, these midline nuclei do not transmit peripheral sensory or motor information to primary sensorimotor cortices nor do they relay information from primary cortices to association cortices like thalamic “association” nuclei. Instead, thalamic midline nuclei receive information from and send information to a large number of cortical and subcortical structures, many of which are considered to be part of the limbic system (Groenewegen & Witter, 2004). Accordingly, midline nuclei have been classified as part of the “limbic thalamus” (Vertes, Linley, & Hoover, 2015). The RE, located just above the third ventricle, is the largest of the four midline nuclei and has recently attracted significant attention. Efferents originating in the RE are almost exclusively directed to “limbic” cortical structures and form a major thalamic input into various temporal and frontal association cortices (Vertes, 2006; Wouterlood, Saldana, & Witter, 1990). In particular, the RE provides the strongest thalamic input to the hippocampal formation, where its excitatory fibers bypass the tri-synaptic loop and the temporoammonic pathway to synapse specifically on distal dendrites of pyramidal cells and interneurons in stratum lacunosum-moleculare of ventral and dorsal CA1 as well as in stratum moleculare of ventral and dorsal subiculum (Dolleman-Van der Weel & Witter, 2000; Vertes et al., 2015; Wouterlood et al., 1990). Besides this strong projection into the hippocampal formation the RE heavily innervates layers 1 and 5/6 of the medial prefrontal cortex (mPFC) with an emphasis on the prelimbic and infralimbic cortices (Vertes, Hoover, Do Vallee, Sherman, & Rodriguez, 2006). Both of these strong efferent pathways are reciprocal and direct synaptic connections between afferents from mPFC and efferents to the hippocampus (HC) have been described (Cassel et al., 2013; Varela, Kumar, Yang, & Wilson, 2014; Vertes, Hoover, Szegeti-Buck, & Leranth, 2007). Accordingly, the RE is thought to represent an important node in hippocampal–prefrontal communication. This prediction based on anatomical connectivity is supported by transient neuronal synchronization across these structures and functional studies showing that inactivation of the RE affects especially those behaviors, which depend on prefrontal–hippocampal communication, such as spatial working memory and long-term spatial and contextual memory consolidation (Ali et al., 2017; Barker & Warburton, 2018; Ferraris et al., 2018; Griffin, 2015; Hallock, Wang, & Griffin, 2016; Hembrook & Mair, 2011; Hembrook, Onos, & Mair, 2012; Roy, Sverson, Mazeh, & Kocsis, 2017; Vetere et al., 2017; Xu & Sudhof, 2013). In addition, the RE has been implicated in attention, behavioral flexibility and impulse control but also more basic functions such as circadian regulation, nociception and fear (see Cassel et al., 2013 for review; Prasad, Macgregor, & Chudasama, 2012; Silva, Burns, & Graff, 2018; Vetere et al., 2017).

Whereas the efferents of the RE are largely restricted to temporal and frontal association cortices, it receives input from a large number of different cortical and subcortical structures, which likely modulate its function and may account for its complex role in cognition. Studies regarding the afferent connectivity of the RE have almost exclusively been performed in rats (Cassel et al., 2013; Cavdar et al., 2008; Herkenham, 1978; McKenna & Vertes, 2004; Vertes, 2002). In light of the unique role of the mouse as a genetically accessible model system for the functional analysis of brain circuits in mammals, we have set out to delineate the afferent connectivity of the mouse RE using retrograde Fluoro-Gold tracing and to compare the results with previous rat studies.

2 | MATERIALS AND METHODS

2.1 | Animals and surgery

All experiments were performed in accordance with the German law on animal protection and approved by the Animal Care and Ethics Committee of the University of Kiel. A total of 15 male and female 4-month old mice with a C57BL/6J genetic background (RRID: MGI:2670020) were used in this study. All mice received a Fluoro-Gold injection into the nucleus reunions. Of these, four mice (one male, three females) were selected in which the spread of the injection was best contained to the RE to obtain the most accurate results. Animals were housed in a temperature-controlled environment with a 12-hr light/dark cycle and free access to food and water. Anesthesia was induced with 3% isoflurane in O2 by inhalation and maintained on 2% isoflurane throughout the surgery. The animals were head fixed in a stereotactic frame (David Kopf Instruments, Tujunga, CA) and body temperature was maintained by a heating mat placed underneath the animal. A vertical incision in the skin was made to expose the skull. A drill hole was made relative to Bregma and 0.1–0.2 μL of 4% Fluoro-Gold™ (FG; Fluorochrome, Denver, CO) dissolved in saline was unilaterally injected over a period of 3 min to bilaterally target the nucleus RE using a Hamilton syringe attached to a 33 gauge blunt metal injection needle. Injection coordinates for the nucleus RE were 0.82 AP/−1.55 LM/−4.5 DV at a 17° angle relative to bregma. After injection the skin was sutured using Ethicon Coated Vicryl (Ethicon Inc., Dülmen, Germany). Mice received an intraperitoneal injection with buprenorphine for postoperative analgesia and were allowed to recover in a warm cage until fully awake.

2.2 | Histology and imaging

After 10–12 days of surgery, animals were deeply anesthetized by intraperitoneal injection of pentobarbital (50 mg per 30 g body weight). The mice were then transcardially perfused with phosphate-buffered saline (PBS, pH 7.4) for 1 min followed by 4% paraformaldehyde (PFA) in PBS for 12 min. Brains were collected, postfixed in 4% PFA, at 4°C for several hours, cryoprotected overnight in a 30% sucrose solution in PBS at 4°C and frozen at –80°C. Brains were cut into 16 μm thick coronal sections on a Leica CM 3050S cryostat (RRID:SCR_016844). Sections were mounted on polylysine-coated slides (Thermo Fisher Scientific, Bremen, Germany) and cover-slipped using Mowiol (Sigma-Aldrich Biochemie GmbH, Hamburg, Germany). Sections were imaged on a Zeiss Axio Imager 2 fluorescence microscope using a 10x objective (EC Plan-Neofluar 10X, Carl Zeiss, Göttingen, Germany) using Zeiss ZEN Imaging Software (RRID:...
SCR_013672). Tiled digital images were captured for each region of interest and analyzed off-line using Adobe Photoshop CS4 (Adobe, Mountain View, CA; RRID:SCR_014199). Coordinates relative to bregma were determined for each section using the Mouse Brain in Stereotactic Coordinates (MBSC; Paxinos & Franklin, second edition). The location and relative density of FG positive cells were determined for each section and documented manually in corresponding coronal plates of the mouse brain atlas (MBSC; Paxinos & Franklin, second edition). Interindividual differences were analyzed and compared regarding injection location and spread to determine the representability of the subject. Occasionally some leakage of FG along the injection tract of the needle was observed. Accordingly, in some animals labeling of structures located in the injection path (e.g., motor and somatosensory cortices, retrosplenial cortex, and dentate gyrus [DG]) could be perceived. Artfactually labeled areas were identified by comparison of ipsi- and contralateral staining. As FG was deposited on both sides of the RE by unilateral injection, true retrograde tracing will affect both hemispheres.

3 | RESULTS

About 10 days after stereotaxic injection of FG into the RE (see Figure 1) we selected four C57Bl/6J mice (one male (A), three females (B, C, D)), 4 months of age, based on location and spread of the FG deposition. In these four mice all injections were located centrally in the anterior/posterior axis of the RE of the midline thalamus (Figures 1 and 2o–5o), with the FG deposit in mouse A spreading more towards the anterior RE and in mouse D more towards the posterior RE. In all animals FG spread to both sides of the RE, with stronger labeling on the injection side. However, in mouse D the injection was largely contained to the ipsilateral side, as was the case in the more posterior sections of mouse A and more anterior sections of mouse C (Figures 2o, 4o, and 5o).

For the detection of traced cells, labeling in the hemisphere contralateral to the injection site was deemed critical to avoid misinterpretations due to artifacts from the injection procedure (see Section 2). In the following, unless specifically stated, labeling is reported if detected bilaterally in at least three out of the four animals (see also Table 1 for details). Data for mouse A–D is depicted in the respective Figures 2–5.

3.1 | Thalamus

In agreement with the dense connections of the RE within the diencephalon reported for other species (Vertes, Linley, & Hoover, 2015), abundant labeling was found in various nuclei of the thalamus. In close proximity to the injection site (Figures 2f, 2–5g, and 2–5h), labeled cells were found in the rhomboid thalamic nucleus (Rh) as well as in the ventral RE of all mice. Further labeling was found in two animals (B, D; Figures 3h and 5h) in the ventromedial thalamic nucleus (VM) and zona incerta (ZI; Figure 6a). The mediodorsal thalamic nucleus showed labeled cells in all subregions (lateral, central, and medial), but only ipsilateral to the injection site. In all mice, labeled cells were also found in the ipsilateral intermediodorsal thalamic nucleus and centromedial thalamic nucleus (CM), although more anterior in A and C.

3.2 | Epithalamus

As the epithalamus lay partially within the injection tract and labeled cells were mainly observed ipsilaterally (Figures 2–5g and 2–5h), staining due to leakage cannot be ruled out. Traced cells in the
habenular nucleus (Hb) were found mainly ipsilaterally, with only one animal (A; Figure 2h) displaying a few cells in the contralateral medial and lateral Hb. Further, modest labeling of cells could be observed for three mice in the lateral nuclei of the septum, the anterior intermediate part (LSI; A, D; Figures 2c and 5c) and septofimbrial nucleus (SF; C, D; Figures 4d,e and 5d,e).
3.3 Hypothalamus

Sparse labeling was found for the hypothalamic area. Only the posterior hypothalamic area (PH) was marked in all mice (Figures 2–5h,i). In the mammillary nuclei, positive cells were found for three mice (A, C, and D) in the lateral, medial and supramammillary nucleus (ML, MM, and SuM), but not for the more rostral laying nuclei, such as the pre mamillary or tuberomammillary nuclei (Figures 2j, 4j, 5j,k, and 6e).

**Figure 3** Rostro-caudally aligned coronal plates illustrating the location of FG-labeled neurons for the side contralateral to the injection side for mouse B. Dots represent the relative density of positively labeled cells. Coordinates according to bregma are depicted below the images. Panel (O) depicts the respective spread at the injection site and along the injection tract for each subject. Plates are modified from the Paxinos and Franklin Mouse Brain Atlas [Color figure can be viewed at wileyonlinelibrary.com]
3.4 | Midbrain and superior colliculus

The rostral part of the ventral tegmental area (VTA) showed retrogradely labeled cells in two animals (A, D; Figures 2j and 5j), but not the caudal part. Also, in some animals, the superior colliculus (SC; A, D; Figures 2j,k and 5j,k) and periaqueductal gray (PAG; A, B, D; Figures 2i–l, 3k, and 5i–l) contained FG-positive cells.
3.5 | Cortex

Fluorescently labeled cells were found in the claustrum (Cl; Figures 2b, 4b, and 5c,d) and the rostral part of the dorsal endopiriform cortex (DEn; Figures 2c,e, 3e, and 5c). The piriform cortex (Pir) was negative, except for sparse labeling in one animal (A; Figure 2d). Cells projecting to the nucleus RE were also found in the ectorhinal (Ect; A, D; Figures 2k and 5k,l) and perirhinal cortex (PRh; A, C, D; Figures 2k, 4k, 5k, and 6d). Sparse labeling of the lateral (n = 2 [C, D; Figures 4k,l and
TABLE 1  

Summary of ipsi- and contralateral labeled areas in the mouse brain produced by injection of Fluoro-Gold in the nucleus reuniens

| Structure                        | Labeling | Ipsi | Contra |
|----------------------------------|----------|------|--------|
| Thalamus                         |          |      |        |
| Rhomboid n.                      | ++       | ++   |        |
| Mediodorsal n.                   | ++       | –    |        |
| Intermediodorsal n.              | +        | –    |        |
| Centromedial n.                  | ++       | –    |        |
| Ventromedial n.                  | ++       | ++   |        |
| Intergeniculate leaflet          | –        | –    |        |
| Lateral dorsal n.                | +        | –    |        |
| Lat geniculate compl. vent       | –        | –    |        |
| Paraventricular n.               | –        | –    |        |
| Reticular n.                     | +        | –    |        |
| Septum                           |          |      |        |
| Medial                           | –        | –    |        |
| Lateral                          | +        | +    |        |
| Septofimbrial nucleus            | ++       | ++   |        |
| Subthalamus                      |          |      |        |
| Zona incerta                     | ++       | ++   |        |
| Hypothalamus                     |          |      |        |
| Anterior hypothalamic area       | –        | –    |        |
| Anterior n.                      | –        | –    |        |
| Dorsomedial n.                   | –        | –    |        |
| Lateral hypothalamic area        | –        | –    |        |
| Mamillary nuclei                 |          |      |        |
| Lateral mammillary n.            | ++       | ++   |        |
| Medial mammillary n.             | ++       | ++   |        |
| Premammillary n.                 | –        | –    |        |
| Supramammillary n.               | ++       | ++   |        |
| Tuberculum mammillany n.         | –        | –    |        |
| Paraventricular n.               | –        | –    |        |
| Posterior hypothalamus           | ++       | ++   |        |
| Ventromedial n.                  | –        | –    |        |
| Midbrain and superior colliculus |          |      |        |
| Ventral tegmental area           | +        | +    |        |
| Superior colliculus              | +        | +    |        |
| Periaqueuctal gray               | ++       | ++   |        |
| Cortex                           |          |      |        |
| Agranular insular                | ++       | ++   |        |
| Claustrum                        | ++       | ++   |        |
| Dorsal peduncular cortex         | ++       | ++   |        |
| Ectorhinal                       | ++       | ++   |        |
| Endopiriform                     | ++       | ++   |        |
| Orbital cortex (MO,VO,LO)        | ++       | ++   |        |
| Perirhinal                       | ++       | ++   |        |

(Continues)

3.6 | Retrosplenial cortex

In the deeper layers of the anterior granular and agranular retrosplenial cortex (RSG and RSA), as well as in the more rostral fused cingulate/retrosplenial area (RS/Cg), labeled cells could be found ipsilaterally in most mice but contralateral only in one mouse (A; Figure 2f,g).

3.7 | Motor, parietal association, and somatosensory cortex

Ipsilateral staining of the lateral and medial parietal association cortex was observed. Also, positive cells were detected bilaterally in the primary and secondary motor cortex (M1 [Figures 2a–g, 4e–g, and 5c–g] and M2 [Figures 2a–g, 3a–e, 4a–d, and 5a–g]), as well as in the primary somatosensory cortex (hind limb region, S1HL; Figures 2f, 4e–g, and 5e–g). However, as these structures lay at the entrance point of our cannula, these signals are possibly caused by FG leakage. The bilateral labeling of the motor and somatosensory cortex at the coordinates of entrance may be a consequence of the extensive contralateral connections of these cortical areas (Mao et al., 2011).
3.8 | Prefrontal cortex

Retrogradely labeled cells from the RE are found abundantly in the mPFC in all mice (Figures 2a–c, 3a–c, 4a–c, 5a–c, and 7a,c). The primary and secondary cingulate cortex (Cg1 and 2; Figure 6b) as well as prelimbic (PrL) and infralimbic (IL) areas showed extensive labeling in deep layers. This labeling extended into the ventral part of the mPFC with positive cells in the deep layers of the medial, ventral and lateral orbital cortex (MO; A, B, D; Figures 2a, 3a, and 5a), VO (Figures 2–5a), LO (A, D; Figures 2a and 5a).

3.9 | Ventral forebrain

FG positive neurons were detected in the deep layers of the dorsal peduncular cortex (DP) and dorsal tenia tecta (DTT). Labeled cells were located also in the deep layers of the ventral and dorsal agranular insular cortex (AI; Figure 2a,b). The caudate putamen (CPu) showed rostro-ventrally abundant labeling, but was marked only ipsilaterally, except for one animal (D; Figure 5b). Due to its variability, we believe labeling here represents an artifact caused by small contaminations of the injection tract.

3.10 | Hippocampus and subiculum

The HC and subiculum showed prominent labeling, especially in the ventral areas.

4 | DISCUSSION

Within this tracing study of afferent projections to the nucleus reuniens in mice, widespread connections throughout the brain were found, which to a large degree recapitulated those described in rat. However, we also found several remarkable differences (Figure 8).

4.1 | Afferent connections corresponding to those reported for rats

As expected based on previous electrophysiological and anterograde tracing studies (Eleore, Lopez-Ramos, Guerra-Narbona, & Delgado-
Garcia, 2011; Xu & Sudhof, 2013), we were able to confirm in mice the extensive projections originating in the prefrontal cortex and HC previously reported in rats (Herkenham, 1978; McKenna & Vertes, 2004), with an origin mainly located in the deep layers of all subareas of the mPFC (including cingulate cortex and prelimbic and infralimbic areas), as well as the ventral CA1 (Figures 2–7). However, the RE not only receives afferents from these structures but also sends strong projections to these structures (Varela et al., 2014). These anatomical findings have been complemented by functional studies stressing the RE as an important hub between mPFC and HC in both mice and rats (Ali et al., 2017; Barker & Warburton, 2018; Ferraris et al., 2018; Griffin, 2015; Hallock et al., 2016; Hembrook et al., 2012; Hembrook & Mair, 2011; Roy et al., 2017; Vetere et al., 2017; Xu & Sudhof, 2013). The directionality and content of the information transfer are subject of ongoing investigation.

Another prominent efferent "limbic" target structure of the RE is the entorhinal cortex (Dolleman-van der Weel, Lopes da Silva, & Witter, 2017; Dolleman-Van Der Weel & Witter, 1996), but no strong reciprocal afferent projections were apparent in our tracings. This lack or sparseness of entorhinal afferents is largely in agreement with data from rats, which only revealed very weak labeling of the entorhinal cortex, with tracer deposition in the rostro-lateral part of the RE (McKenna & Vertes, 2004). The two mice, which showed labeling in the contralateral entorhinal cortex (C and D) did have the most lateral injection location within
the RE (although ipsilateral). However, as these two animals also had mild tracer leakage along the injection tract in the CA3 region, RE-independent tracing of the entorhinal cortex cannot be ruled out at this point (Goldowitz, White, Steward, Lynch, & Cotman, 1975).

Retrogradely labeled cells were found in the lateral, medial and supramammillary nucleus in three mice (A, C, and D) (Herkenham, 1978; McKenna & Vertes, 2004). The mammillary bodies play a crucial role in mnemonic processes (Magloczky, Acsady, & Freund, 1994; Nelson & Vann, 2017; Vann & Nelson, 2015) and the supramammillary nucleus provides theta-rhythmic inputs to the mPFC, RE, and HC in rats. Our results confirm data from rats of an anatomical substrate for information flow from supramammillary nucleus to RE in mice.

Although the VTA is known in rats to have reciprocal connections with the RE (Beckstead, Domesick, & Nauta, 1979; Herkenham, 1978;
McKenna & Vertes, 2004), only two mice (A, D) in our study showed labeling of the rostral VTA. Similar to the results obtained in rats (McKenna & Vertes, 2004), the lateral septum displayed higher numbers of retrogradely labeled neurons than the medial part.

Several sources show that the RE has reciprocal connections with the superior colliculus (SC). For the afferent connections, Krout, Loewy, Westby, and Redgrave (2001) showed in rat that specifically the RE afferents mainly come from the lateral sector of the intermediate gray (InG) and white layers of the SC, similar to what we found for mice. Although our results confirm the connection from the SC to the RE in mice, not all animals (only A, D) showed labeling in this area. Interestingly, a lower degree of labeling of the brainstem and PAG was found in our study compared to rat data (Krut, Belzer, & Loewy, 2002; Krout & Loewy, 2000). Especially more caudally, the few labeled cells we do find lie mainly in the dorsal raphe nucleus (DRD) and laterodorsal tegmental nucleus (LDTg; n = 1 [A]), whereas in rats, several other nuclei, including the dorsal periaqueductal gray, pretectum, and monoaminergic nuclei show distinct labeling. Even though the more posterior regions of the brainstem were not investigated in our study, these obvious differences indicate a more extensive connectivity of the brainstem to the RE in rats than in mice, possibly indicating differences in the networks involved in sensory processing and alertness.

Interestingly, for the VTA and the SC, but also the LO, Ect, and LSI, the same two animals, namely A and D, show neurons projecting to the RE. These are all structures known to project to the RE in rat studies. Accordingly, the question is why we did not find any labeled cells in the other two animals (B and C). As mice B and C display an injection spread that lies in between that of animals A and D, subarea differences along the rostro-caudal axis are not a likely cause. Subject A and D, however, did show a more pronounced spread into the ventral RE, possibly explaining difference in labeling.

No significant labeling of the nucleus accumbens (Acb) was found. This is in agreement with previous negative results for the Acb in retrograde tracing studies of the RE in rats (McKenna & Vertes, 2004), although contradicting results have been found across different experiments and species for accumbens-thalamic projections (Koikegami, Hirata, & Oguma, 1967; Powell & Leman, 1976; Swanson & Cwan, 1975; Williams, Crossman, & Slater, 1977). Also, no efferent connectivity with the accumbens is present, as anterograde tracing of the midline thalamic nuclei showed that thalamo-accumbens neurons originate in the paraventricular thalamic nucleus and CM, but not the RE (Su & Bentivoglio, 1990).

### 4.2 | Afferent connections deviating from those reported for rat

The afferent connections reported here for mice are largely in agreement with those previously reported in rats. However, several regions deviated from previous rat data (Figure 8).

In the ventral forebrain we found distinct labeling of the lateral and ventral orbital cortices, as well as the dorsal peduncular cortex (Figures 2–5a and 2–5b). McKenna and colleagues do not describe these regions as containing afferent projections to the RE. However, comparing the figures (McKenna & Vertes, 2004) with the anatomical structures as provided by the MBSC (Paxinos & Franklin, second edition) suggests that indeed these regions do contain positively marked cells also in rats. This further stresses the link of the RE with networks involved in decision making and reward-related signals (Gourley et al., 2013; Ward, Winiger, Kandel, Balsam, & Simpson, 2015).

For the dorsal endopiriform nucleus, most animals showed labeling (A, B, and D); however, with a distinct variation in the rostral to caudal spread (Figures 2c,e, 3e, and 5c). Reciprocal connections between the endopiriform nucleus and the thalamus (mediodorsal and midline nuclei including the RE) have been described before in rodents (Kowianski, Lipowska, & Morys, 1999), however not by retrograde tracing studies of the RE. Future studies will have to investigate the significance of these findings specifically for mice.

Further, we did not find labeled cells in the bed nucleus of the stria terminals, the habenular nuclei, the preoptic area, and the premammillary nuclei as well as several thalamic nuclei, such as the lateral geniculate complex, paraventricular nucleus, the intergeniculate leaflet, and the laterodorsal nucleus (McKenna & Vertes, 2004), although for some of the structures a few subjects did show weak labeling. These results comply with a very weak connectivity for these structures (McKenna & Vertes, 2004), but could also be due to subarea specific projections in the anterior/posterior extend of the RE.

Previous results from rats have been contradictory as to whether the substantia nigra (SN) projects to the RE (Beckstead et al., 1979; Herkenham, 1978; McKenna & Vertes, 2004). We found no evidence of afferent connections from the pars compacta or pars reticulata in mice.

In rats wider retrograde labeling in hypothalamic nuclei has been reported, including the anterior and lateral hypothalamic area and the anterior, dorsomedial, and ventromedial nuclei, as well as the premammillary and tuberomammillary nuclei (McKenna & Vertes, 2004). Of these, the premammillary nucleus, anterior hypothalamic nucleus and ventromedial nucleus constitute the medial hypothalamic defensive system (Canteras, 2002), involved in the processing of threats.

Another key difference between our study and previous data in rat (Herkenham, 1978; McKenna & Vertes, 2004) is that we found no afferents from the amygdala (Figure 6c). Studies in rats on amygdalar efferents have reported sparse projections from the amygdala reaching the medial part of the RE (Pardo-Bellver, Cadiz-Moretti, Novejarque, Martinez-Garcia, & Lanuza, 2012; Su & Bentivoglio, 1990). As our tracer covered this region, it is unlikely that this difference is due to tracer deposition. Retrograde tracing, on the other hand, has indicated that rostrotemporal areas of RE receive amygdalar inputs (McKenna & Vertes, 2004). This part of the RE was injected only in mouse A. However, also this mouse was devoid of labeled cells in the amygdala both ipsi- and contralaterally. Such species-specific differences in neuronal circuitry may contribute to interspecies differences in amygdalar function (Lugo, Smith, & Holley, 2014).

In summary, we have shown that the RE in mice, like in rats, receives inputs from a large number of cortical and subcortical...
structures. Comparing our findings to similar studies in rats, we find a very high level of similarity in the afferents of the RE from mPFC and HC, with projections originating in the deep layers of all subareas of the mPFC, the ventral CA1, and the subiculum. However, we also observed several interesting differences in afferent connectivity. In particular, our results suggest interspecies differences regarding the integration of the RE in circuits of fear, aversion, and defense, with no obvious afferents from the amygdala and the medial hypothalamic defensive system in mice. Such differences have to be taken into consideration for the design of experiments focused on the function of the respective neuronal networks.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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