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Organization of the Sensory Input to the Telencephalon in the Fire-Bellied Toad, *Bombina orientalis*

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ABSTRACT

The functional organization of sensory activity in the amphibian telencephalon is poorly understood. We used an in vitro brain preparation to compare the anatomy of afferent pathways with the localization of electrically evoked sensory potentials and single neuron intracellular responses in the telencephalon of the toad *Bombina orientalis*. Anatomical tracing showed that the anterior thalamic nucleus innervates the anterior parts of the medial, dorsal, and lateral pallia and the rostralmost part of the pallium in addition to the subpallial amygdala/ventral pallidum region. Additional afferents to the medial telencephalon originate from the thalamic eminence. Electrical stimulation of diverse sensory nerves and brain regions generated evoked potentials with distinct characteristics in the pallium, subpallial amygdala/ventral pallidum, and dorsal striatopallidum. In the pallium, this sensory activity is generated in the anterior medial region. In the case of olfaction, evoked potentials were recorded at all sites, but displayed different characteristics across telencephalic regions. Stimulation of the anterior dorsal thalamus generated a pattern of activity comparable to olfactory evoked potentials, but it became similar to stimulation of the optic nerve or brainstem after bilateral lesion of the lateral olfactory tract, which interrupted the antidromic activation of the olfactohabenular tract. Intracellular bimodal sensory responses were obtained in the anterior pallium, medial amygdala, ventral pallidum, and dorsal striatopallidum. Our results demonstrate that the amphibian anterior pallium, medial amygdala/ventral pallidum, and dorsal striatopallidum are multimodal sensory centers. The organization of the amphibian telencephalon displays striking similarities with the brain pathways recently implicated in mammalian goal-directed behavior. J. Comp. Neurol. 502: 55–74, 2007.

Indexing terms: evoked potentials; amphibians; pallium; amygdala; striatopallidum

Reports of diverse sensory-evoked potentials and sensory-driven units in the pallium of anuran amphibians revealed that the latter structure is involved in multiple sensory functions besides olfaction (reviewed in Kicliter and Ebbesson, 1976; Mudry and Capranica, 1980). These studies described in general terms the sites of sensory responses (medial or dorsal pallium, medial telencephalic wall). Few investigators have studied evoked activity in the amphibian striatum (vision: Gruberg and Ambros, 1974; audition: Mudry and Capranica, 1980; electrosensation: Northcutt and Plassmann, 1989; Birkhofer et al., 1994), which is known to receive input from the sensory thalamus in parallel with the pallium (Scalia, 1976; Kicliter, 1979; Wilczynski and Northcutt, 1983a; Neary, 1984; Wicht and Himstedt, 1988; Roth and Grunwald, 2000; Roth et al., 2003). The most detailed evoked potential studies showed that most of the dorsal surface of the pallium is activated by visual and somatosensory input from the dorsal thalamus (Supin and Gusel'nikov, 1965; Karamian et al., 1966; Vesselkin et al., 1971). However, a systematic study of the distribution of sensory-evoked responses in the amphibian telencephalon is lacking. It is

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noteworthy that previous functional studies did not include the amygdala—an additional telencephalic structure of known importance in sensory processing in amniotes. This paucity of data makes difficult an assessment of the amphibian telencephalon in an evolutionary framework.

Northcutt (2006) noted from anatomical observations that the teleost pallium appears to comprise regions corresponding to the hippocampus and amygdala, i.e., multimodal limbic structures. However, Prechtl et al. (1998) found a mixture of unimodal and multimodal sensory regions in the pallium of a mormyrid fish, resembling the sauropsid/mammalian pattern of cortical organization (Krubitzer et al., 1995; ten Donkelaar, 1998a). Similarly, Yamamoto and Ito (2005) suggested that the sensory pathways from the preglomerular complex target distinct zones of the dorsal telencephalon in cyprinid fishes. Therefore, the question is: Are unimodal sensory regions hidden within the amphibian pallium or is it entirely multimodal? A theory of the amphibian pallium could help explain the role of expanding sensory thalamic input to the pallium in amniote vertebrates (Butler, 1994; Striedter, 1997). Such a theory could also provide a simplified model to understand the function of the mammalian cortex. In the case of the amygdala, functional data are dearly needed in amphibians in order to clarify current controversies resulting from different interpretations based on anatomical data (Laberge et al., 2006; Moreno and González, 2006). Finally, little has been written on the possible roles of the two parallel sensory pathways to the amphibian telencephalon, which differ from the amniote situation in that they are by and large isolated from each other. Wilczynski and Northcutt (1983b) proposed that the striatum organizes multimodal sensory information involved in attention and orientation behavior, and Belekhova (1990; cited in ten Donkelaar, 1998b) proposed that the medial pallium processes 'limbic' sensory information possibly involved in defensive and foraging behavior. Veenman et al. (1989), on the premise that the telencephalon is responsible for the ability to make decisions in new situations, postulated the existence of two parallel sensory systems in frogs acting in the pallium and striatum. Their striatal sensorimotor system would determine the different behavioral possibilities associated with a stimulus, while their pallial selection system would exert control over which behavior is chosen from the different possibilities.

In the present study we used electrophysiological recordings to map sensory evoked activity in the telencephalon of the fire-bellied toad, *Bombina orientalis,* and describe previously unknown details of the anatomy of the diencephalic pathways to the telencephalon. The results suggest that each neuron in the telencephalic sensory centers outside the olfactory pallium receives multimodal sensory input. In the case of the pallium and amygdala, sensory input is likely in the form of integrated multimodal information relayed from the anterior thalamic nucleus, as suggested by Roth et al. (2003) and Westhoff et al. (2004), in addition to a separate olfactory input, whereas in the case of the dorsal striatopallidum, thalamic input originates from the posterior dorsal region.

MATERIALS AND METHODS

A total of 31 adult fire-bellied toads, *B. orientalis,* were used for experimentation. The animals were bought from a local supplier (Tropenhaus, Hamburg, Germany). Seven of them were used for biocytin tract-tracing and 24 for electrophysiology. The experimental procedures were approved by the veterinary office of the Ministry of Health of the State of Bremen, Germany. All experiments were carried out in vitro in isolated brain preparations. After deep anesthesia by exposure to a solution of 0.5% tricaine methane sulfonate, the animals were quickly decapitated, the lower jaw was removed, and the skull was opened from the roof of the mouth to enable brain dissection. The dissection was performed in Ringer's solution consisting of Na^+ 129 mM, K^+ 4 mM, Ca^{2+} 2.4 mM, Mg^{2+} 1.4 mM, Cl^- 115 mM, HCO_3^- 25 mM, glucose 10 mM, perfused with 95% O_2/5% CO_2 until a pH of 7.3 was measured. For electrophysiology, the isolated brain was kept in Ringer's solution at 4°C between experimental days and could typically be used for experimentation during a whole week.

Electrophysiological recordings

For recordings, the brain was pinned down at the bottom of a recording chamber equipped with an overlooking dissecting microscope and continuously perfused with Ringer's solution (6 mL/min) at a temperature of 14–18°C. Evoked potentials were measured using glass micropipettes filled with a solution of 2 M NaCl with the tip cut at a diameter of ≈10 μm. Intracellular recording and labeling were made with glass micropipettes filled with 2% biocytin (Sigma-Aldrich, St. Louis, MO) dissolved in 0.3 M KCl following a procedure previously described in Laberge and Roth (2005), with the exception that most recordings were made in intact brains. The impedance of the intracellular electrodes ranged from 100–200 MΩ. A silver wire pinned on the floor of the recording chamber served as reference electrode. Electrical potential was measured with a differential electrometer (Duo 773, World Precision Instruments [WPI], Sarasota, FL) connected to an A/D interface (micro 1401mkII, Cambridge Electronic Design, Cambridge, UK) and operated from a computer using the Signal 2.15 data acquisition program (CED). A feature of the Duo 773 was used to amplify intracellular signals 10 times, while evoked potential signals were amplified through custom-built 100× battery-powered amplifiers. The setup enabled recording of two evoked potentials or one intracellular response and one evoked potential simultaneously, as well as the use of two stimulation electrodes at once.

Artificial stimulation of sensory afferents was achieved in two ways: concentric stimulation electrodes (Model TM33CCINS 3″, tungsten core electrode of 4 μm at the tip separated from an insulated stainless steel tube conductor by a distance of 0.4 mm, WPI) for brain sites and custom-built suction electrodes for nerves. Custom-built stimulators triggered digitally were used to deliver current pulses between 0.01–1.5 mA. The sites and nerves stimulated in the present study are the following: medulla oblongata (MO; likely involves modalities relayed through this region like somatosensory, taste, visceral, auditory, and vestibular sensations) in the obex region or central gray, torus semicircularis (Torus; involves both somatosensory and auditory sensations), anterior dorsal thalamus (DT; likely involves all sensory modalities, except olfaction), optic nerve (OpN; vision), olfactory/vomeronasal nerve bundle (ON/VN; involves both main olfactory and vomeronasal afferents), dorsal root of the second spinal nerve (2Sp; somatosensory), glossopharyngeal-vagal-accessory

nerve bundle (IX-X-XI; taste, visceral, and somatosensory sensations), octaval nerve (VIII; auditory and vestibular sensations), and trigeminal nerve (V; somatosensory). Apart from threshold measurements, the stimulation currents used in the experiments were as follows: MO (0.2, 0.5, or 1.0 mA), Torus (0.5 or 1.0 mA), OpN (0.1 or 0.2 mA), ON/VN (0.1 or 0.2 mA), and 0.05–1.5 mA for the additional stimulation sites.

For the principal evoked potential experiments, recordings were obtained directly below the surface from as many as 13 dorsal and 15 ventral telencephalic sites successively on each side of the brain with stimulation of the MO, OpN, and ON/VN. Evoked potentials were measured at intervals of 2 seconds or 1 minutes and repeated at least 5 times when a response was detected or 2 times when no response was seen. The same experiments were performed with Torus and DT stimulation. However, ventral responses could not be obtained in these cases because the stimulation sites proved inaccessible in that configuration. Thus, DT responses were obtained from a lateral approach after longitudinal bisection of the brain. Note that the Torus was inaccessible from a lateral approach. DT and toral responses were additionally obtained from six medial sites by turning the brain halves on the other side. The recording sites were spaced evenly along the longitudinal axis of the brain and their position was guided by the inspection of landmarks present in the telencephalon across animals. The recording electrode was moved with the help of a hydraulic three-axis micromanipulator (model ONO-131, Narishige, Tokyo, Japan). Efforts were made to randomize recording site sequences. Recording at various depths was also made in the medial pallium by inserting the recording electrode ventrally from the dorsal surface by steps of 100 μm. No histological labeling of the evoked potential recording sites was performed in order to enable the recording of responses to multiple sensory stimuli in the same brain during the course of an experimental week. This effectively minimized the number of animals used and the variation resulting from intersubject differences in response. The necessity of histological labeling of recording sites was thus entrusted to intracellular labeling.

Additional evoked potential experiments involved recording of responses to stimulation of additional sensory stimuli in the anterior medial pallium as well as simultaneous two-channel recording in the pallium to measure the flow of DT, OpN, and MO evoked potentials with precision. The latter type of experiment was achieved by moving one recording electrode away or toward the anterior medial pallium along the longitudinal or latitudinal axis of the brain in steps of 250 μm within the pallium. Evoked potentials were averaged before measures of latency of response and latency to response peak were obtained with the help of the program Signal (CED). Differences in conduction velocity between stimulation sites were tested with a two-tailed Student's test with alpha set at 0.05. In an additional experiment, summation of sensory evoked potentials was achieved by delaying one of the two stimulations involved so that the response peaks would coincide in time. The same principle was followed for summation of intracellular responses.

In the case of toral stimulation, the electrode had to be inserted into the brain between the cerebellum and the caudal optic tectum at a slight angle aimed rostrally. Brain lesions were made in two brains to ascertain the site of stimulation by applying a 2-mA constant current for 5 seconds. Bilateral lateral olfactory tract (LOT) lesions were made to eliminate the antidromic olfactory component evoked by stimulation of the olfactohabenular tract, which passes through the anterior dorsal thalamus (Roth et al., 2004). In that case, lesions were made by cutting the lateral wall of the caudal telencephalon between the dorsal pallium and the lateral amygdala with fine scissors, leaving the medial region intact. The extent of these brain lesions was visualized on transverse sections counterstained with cresyl violet. The photomicrographs were scanned with a digital camera (AxioCam HR, Carl Zeiss) and graphically processed for optimal contrast using Photoshop (Adobe Systems, San Jose, CA).

Biocytin tract-tracing

Small crystals of biocytin were applied to the medial surface of the anterior diencephalon in longitudinally split brains. The biocytin application method and histological procedure has been described in detail in Laberge and Roth (2005). The labeling and outlines of brain sections were charted with the help of a camera lucida. Photomicrographs were made as described above.

RESULTS

Sensory-evoked potentials

An overview of the mapping of evoked responses in the telencephalon of *B. orientalis* obtained with stimulation of the MO, Torus, OpN, and ON/VN is presented in Figures 1–4. The anatomical guidelines used in the following section are represented schematically in the left dorsal and right ventral views in Figure 1. Reliable responses were measured across animals with evoked potentials displaying specific characteristics depending on the sensory stimulus. At 1-minute interstimulus intervals the evoked responses in the pallium generated by MO (Fig. 1A) or toral (Fig. 2A) stimuli displayed one or two positive peaks often followed by an oscillation of low amplitude (more so with the MO stimulus) lasting up to 1,000 ms. The evoked responses in the rostralmost pallium, however, were of negative polarity. The long-lasting component habituated quickly with the 2-second interstimulus interval. Moreover, at this short interstimulus interval the MO response usually disappeared completely, whereas the fast peak response obtained with the Torus stimulus persisted, albeit at a lower amplitude. Evoked MO responses were also recorded in the medial amygdala and dorsal striatopallidum (Fig. 1B) where they displayed a fast-descending negative peak, occasionally followed by a negative potential of low amplitude lasting up to 300 ms. Responses obtained by stimulation of the obex region and central gray were similar, except that reliable responses were more difficult to obtain with the central gray stimulus and had a slightly lower latency. Thus, the obex region stimulus was mostly used in the present study. Negative evoked Torus responses were also recorded in the medial amygdala (lower trace in Fig. 2B), but could not be studied in the dorsal striatopallidum due to experimental constraints (see Materials and Methods). Response latencies ranged between 40–70 ms in the anterior medial pallium, 20–45 ms in the medial amygdala, and 35–50 ms in the dorsal striatopallidum. Figure 2C shows the histological examination of one Torus stimulation site produced by electrical lesion.

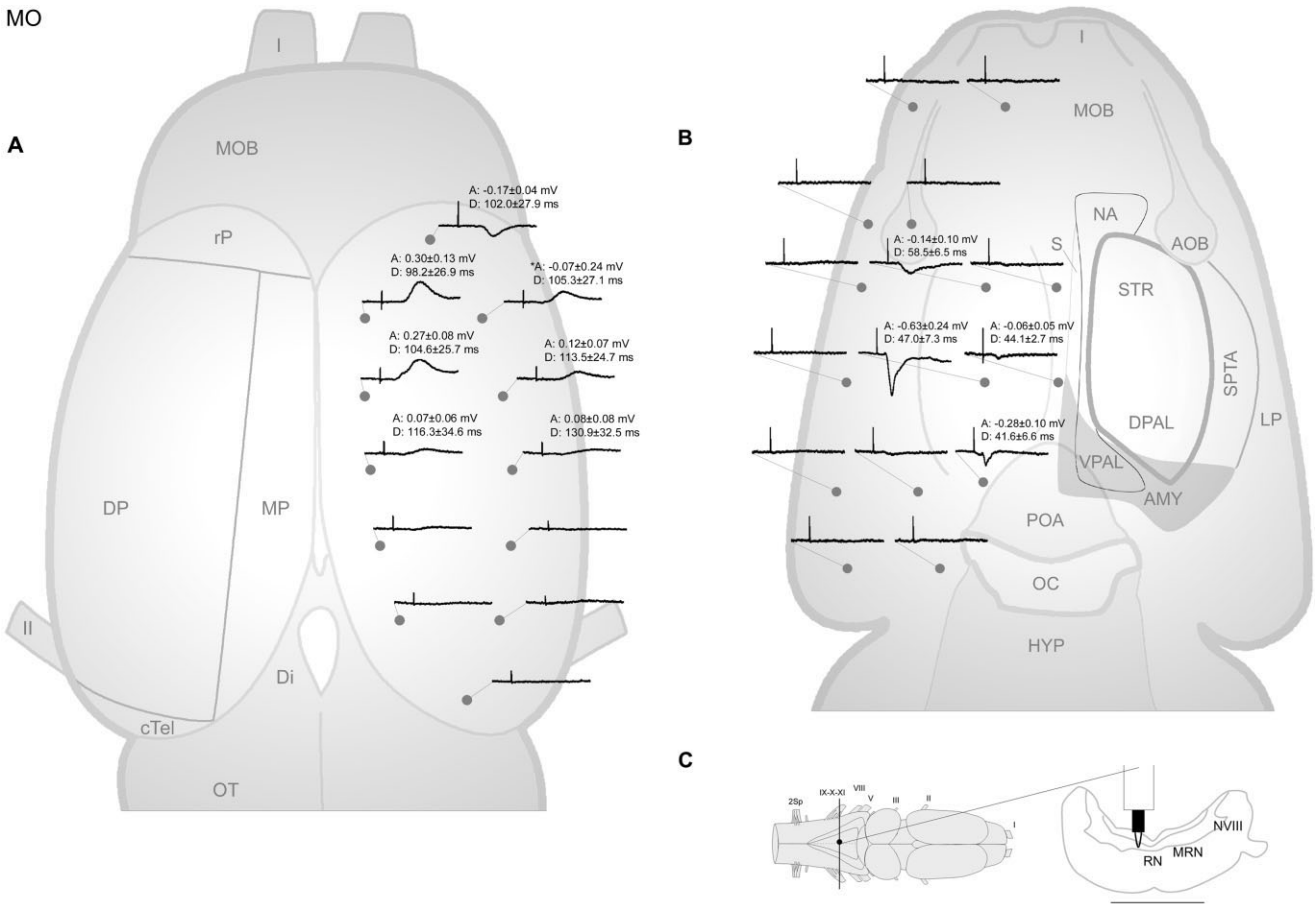


Fig. 1. Evoked potentials measured after stimulation of the median medulla oblongata in the fire-bellied toad. **A:** Dorsal view of the telencephalon. **B:** Ventral view of the telencephalon. The traces shown were recorded in one animal and each trace represents an average of five responses, or two stimuli in case no response was observed. Recording sites are indicated on the schemata by gray dots. The values of response amplitude and delay to peak response for all animals are summarized above each trace (n = 3–5 in A and 4–5 in B). Values (means ± SD) are shown only for sites where responses could be detected in two or more animals. Prestimulus time is 50 ms followed by a 0.7 ms stimulus and a 200 ms poststimulus period. The stimulus artifact in the trace on top of each panel represents amplitude of 0.5 mV. The asterisk indicates that responses of both positive and negative polarity were recorded from that site. The major telencephalic structures are schematically drawn on the left side of the dorsal view and on the right side of the ventral view. **C:** Location of the stimulating electrode. A schematic dorsal view of the brain is shown on the left as well as a transverse brain section on the right to illustrate the stimulation site used to obtain the traces shown in A and B. The scale bar on the right is 1 mm. I, olfactory nerve; II, optic nerve; III, oculomotor nerve; V, trigeminal nerve; VIII, octaval nerve; IX-X-XI, glossopharyngeal-vagal-accessory nerve bundle; 2Sp, second spinal nerve; AMY, amygdala; AOB, accessory olfactory bulb; cTel, caudal pole of the telencephalon; Di, diencephalon; DP, dorsal pallium; DPAL, dorsal pallidum; HYP, hypothalamus; LP, lateral pallium; MO, medulla oblongata; MOB, main olfactory bulb; MP, medial pallium; MRN, medial reticular nucleus; NVIII, octaval nucleus; NA, nucleus accumbens; OC, optic chiasma; OT, optic tectum; POA, preoptic area; RN, raphe nucleus; rP, rostral pallium; S, septum; SPTA, striatopallial transition area; STR, striatum; VPAL, ventral pallidum.

Evoked pallial responses generated by OpN stimulation displayed one positive peak sometimes followed by a low amplitude potential lasting up to 800 ms (Fig. 3A). Again, responses of negative polarity were recorded in the rostralmost pallium. OpN evoked responses were also recorded in the medial amygdala and dorsal striatopallidum (Fig. 3B). In the amygdala, positive evoked potentials of small amplitude rose slowly and persisted for a long period, not showing distinct peaks as in other brain regions. Fast-descending negative peaks lasting 80–100 ms characterized the OpN responses in the dorsal striatopallidum. Note that the apparent response recorded in the vicinity of the optic chiasm is an artifact caused by current spread from the suction electrode situated on the other side of the brain. OpN responses habituated quickly with the 2-second interstimulus interval. Response latencies ranged between 85–111 ms in the anterior medial pallium (average of 96.0 ms contralateral and 104.6 ms ipsilateral), 15–47 ms in the medial amygdala (average of 31.4 ms contralateral, but could not be measured ipsilateral), and 42–83 ms in the dorsal striatopallidum (average of 52.6 ms contralateral and 76.0 ms ipsilateral).

Evoked responses generated by ON/VN stimulation could be measured in the entire telencephalon (Fig. 4A,B). However, the response type varied greatly across recording sites. Evoked responses in the anterior pallium generally displayed two distinct peaks, the earlier of smaller amplitude, whereas responses in the caudal pallium (ex-

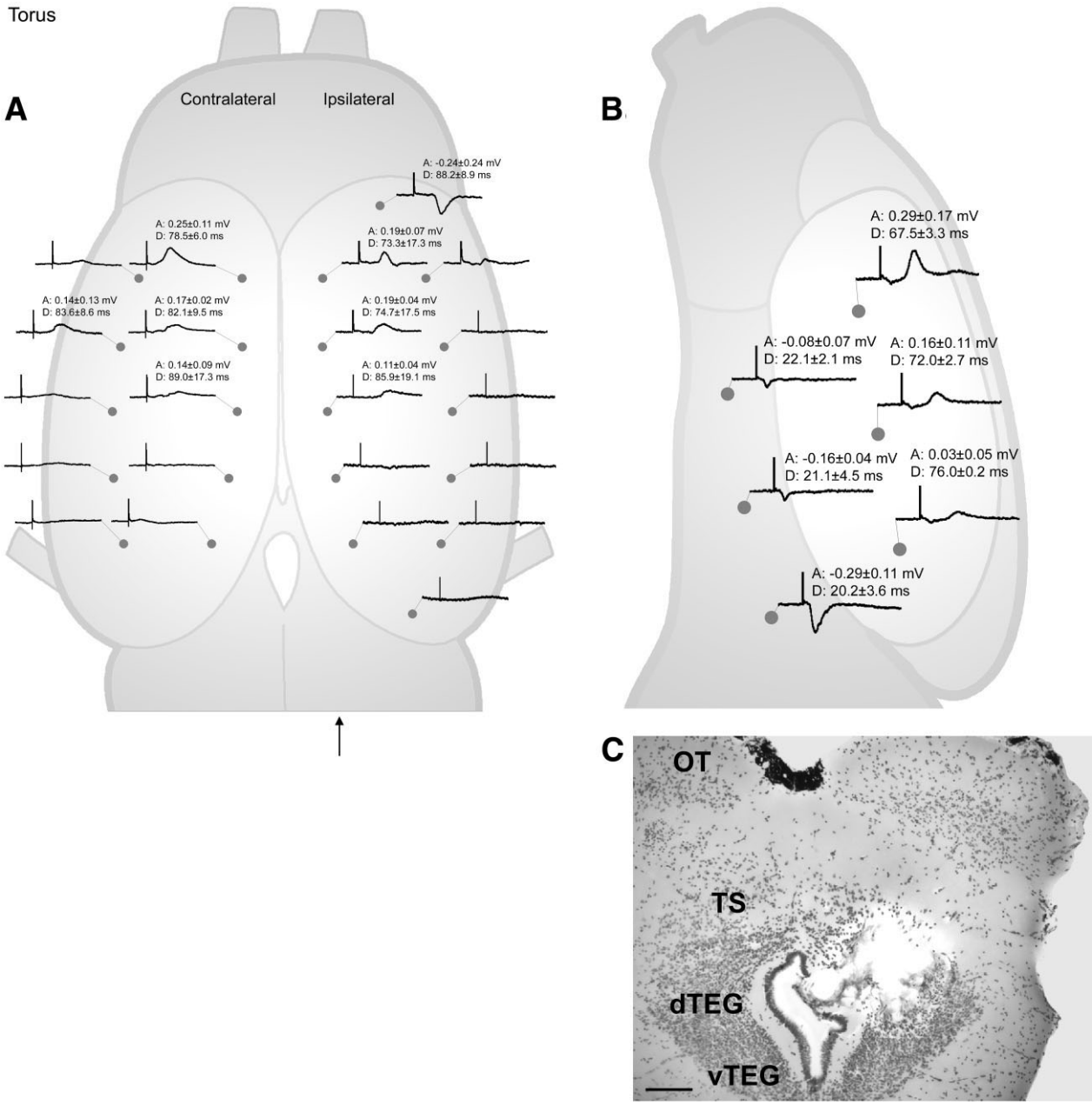


Fig. 2. Evoked potentials measured after stimulation of the torus semicircularis in the fire-bellied toad. **A:** Dorsal view of the telencephalon showing responses to ipsilateral (right) and contralateral (left) stimulation of the torus. **B:** Medial view of the telencephalon showing responses to ipsilateral stimulation of the torus. The traces shown were recorded in one animal and each trace represents an average of five responses, or two stimuli in case no response was observed. Recording sites are indicated on the schemata by gray dots. The values of response amplitude and delay to peak response for all animals are summarized above each trace (n = 2–4 in A and 5 in B). Values (means ± SD) are shown only for sites where responses could be detected in two or more animals. Prestimulus time is 50 ms followed by a 0.7 ms stimulus and a 200 ms poststimulus period. The stimulus artifacts represent amplitude of 0.5 mV. **C:** Photomicrograph of a transverse brain section showing an example of lesion of the torus generated through the same electrode used for generation of telencephalic evoked potentials. Dorsal is upward. The lesion corresponds approximately to the position of the arrow in A. OT, optic tectum; dTEG, dorsal tegmentum; vTEG, ventral tegmentum; TS, torus semicircularis. Scale bar = 500 μm.

cept medial pallium) displayed a large positive peak followed by a negative peak, sometimes of great amplitude. Responses of greater amplitude were recorded in the anterior medial pallium compared to the caudal medial pallium, whereas the responses in the dorsal and lateral pallia displayed greater amplitude in their respective caudal regions. ON/VN evoked responses in the amygdala rose slowly and had low amplitudes. They sometimes displayed a sharp negative peak within the rising phase that did not affect the general trend of the positive potential.

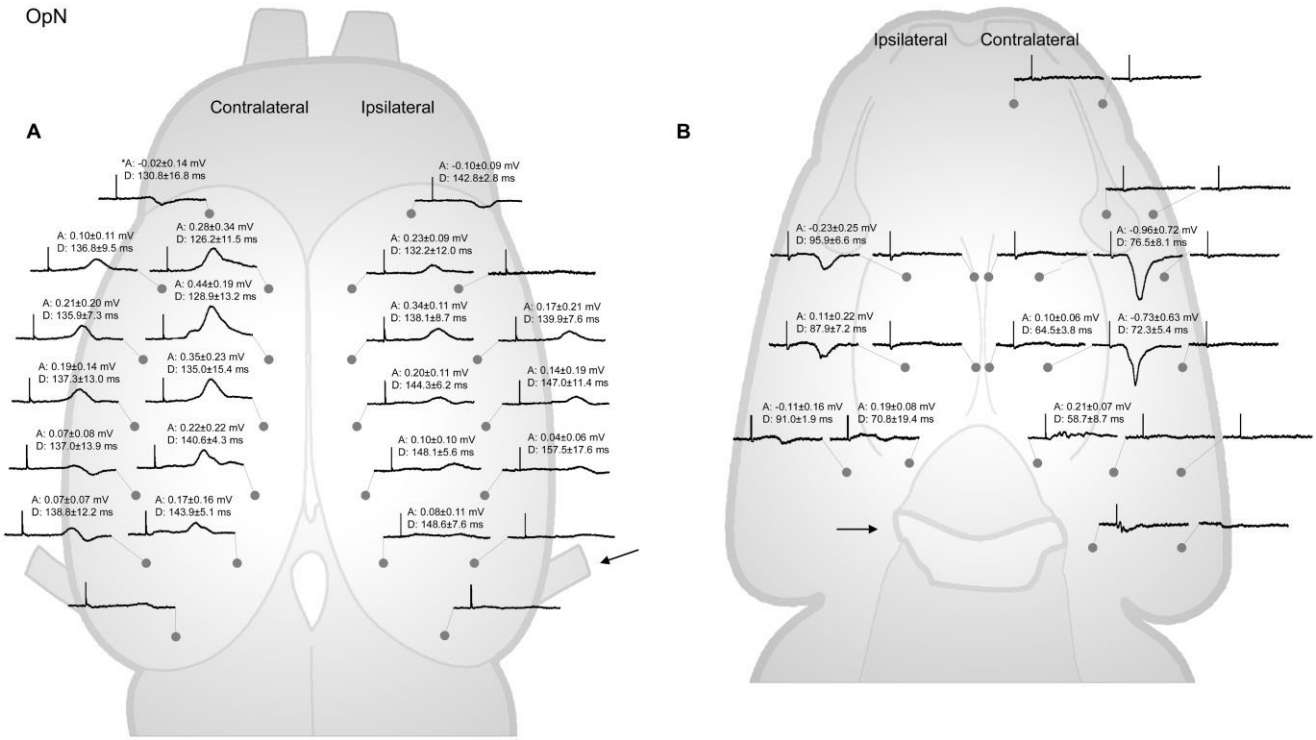


Fig. 3. Evoked potentials measured after stimulation of the optic nerve in the fire-bellied toad. **A:** Dorsal view of the telencephalon showing responses to ipsilateral (right) and contralateral (left) stimulation of the optic nerve. **B:** Ventral view of the telencephalon showing responses to ipsilateral (left) and contralateral (right) stimulation of the optic nerve. Arrows show the location of the stimulating electrode. The traces shown were recorded in one animal and each trace represents an average of five responses, or two stimuli in case no response was observed. Recording sites are indicated on the schemata by gray dots. The values of response amplitude and delay to peak response for all animals are summarized above each trace (n = 3–5 in A and B). Values (means ± SD) are shown only for sites where responses could be detected in two or more animals. Prestimulus time is 50 ms followed by a 0.7 ms stimulus and a 250 ms poststimulus period in A or 200 ms in B. The stimulus artifacts represent amplitude of 0.5 mV. The asterisk indicates that responses of both positive and negative polarity were recorded from that site.

Evoked responses in the dorsal striatopallidum were marked by a fast small positive potential upon which a large and wide peak lasting up to 300 ms developed. Responses in the caudal pallidum were further characterized by very large amplitude and the frequent presence of two sharp indentations in the high amplitude part of the response. ON/VN responses habituated quickly with the 2-second interstimulus interval. Response latencies ranged between 17–42 ms in the anterior medial pallium (average of 28.3 ms contralateral and 25.6 ms ipsilateral), 23–35 ms in the medial amygdala (average of 28.3 ms contralateral, 28.6 ms ipsilateral), and 24–49 ms in the dorsal striatopallidum (average of 35.0 ms contralateral and 31.3 ms ipsilateral).

The responses obtained in the main olfactory bulb after ipsilateral ON/VN stimulation displayed a very fast positive potential, which was difficult to discern from the stimulation artifact, followed by a high amplitude sharp negative peak and oscillation of the potential below resting voltage. These responses were comparable to recordings obtained in the granule cell layer of the bullfrog main olfactory bulb upon olfactory nerve stimulation (Jahr and Nicoll, 1981). On the side contralateral to stimulation, however, the fast positive peak was easily discerned due to its longer response latency, and the following negative peak was much diminished in amplitude compared to the ipsilateral side. A series of positive potential peaks was recorded in the ipsilateral accessory olfactory bulb after ON/VN stimulation, which contrasted with the single positive potential response on the contralateral side.

Response latency proved more difficult to measure than the latency to peak in response due to occasional slow rising early phases of the evoked potentials, which made the beginning of the response hard to discern. Figures 1–3 include only the results for latency to the first peak in the response as well as the maximal amplitude of that peak. Figure 4 in addition lists these values for two response peaks when they were clearly distinguished. Response latencies are measured precisely in an additional experiment described below.

The responses recorded in the deep parts of the medial pallium were similar to those recorded just below the surface with ON/VN stimuli. For the MO, Torus, and OpN the polarity of the evoked potentials was reversed in the deep regions in half of the cases. These reversals depended on the orientation of the electrode track in the medial pallium since the phenomenon correlated exactly across sensory modalities when MO, Torus, or OpN responses were measured in the same electrode track. The potential reversals occurred between depths of 400–600 μm from the surface of the medial pallium. However, electrode penetration tracks were not marked; therefore, no clear

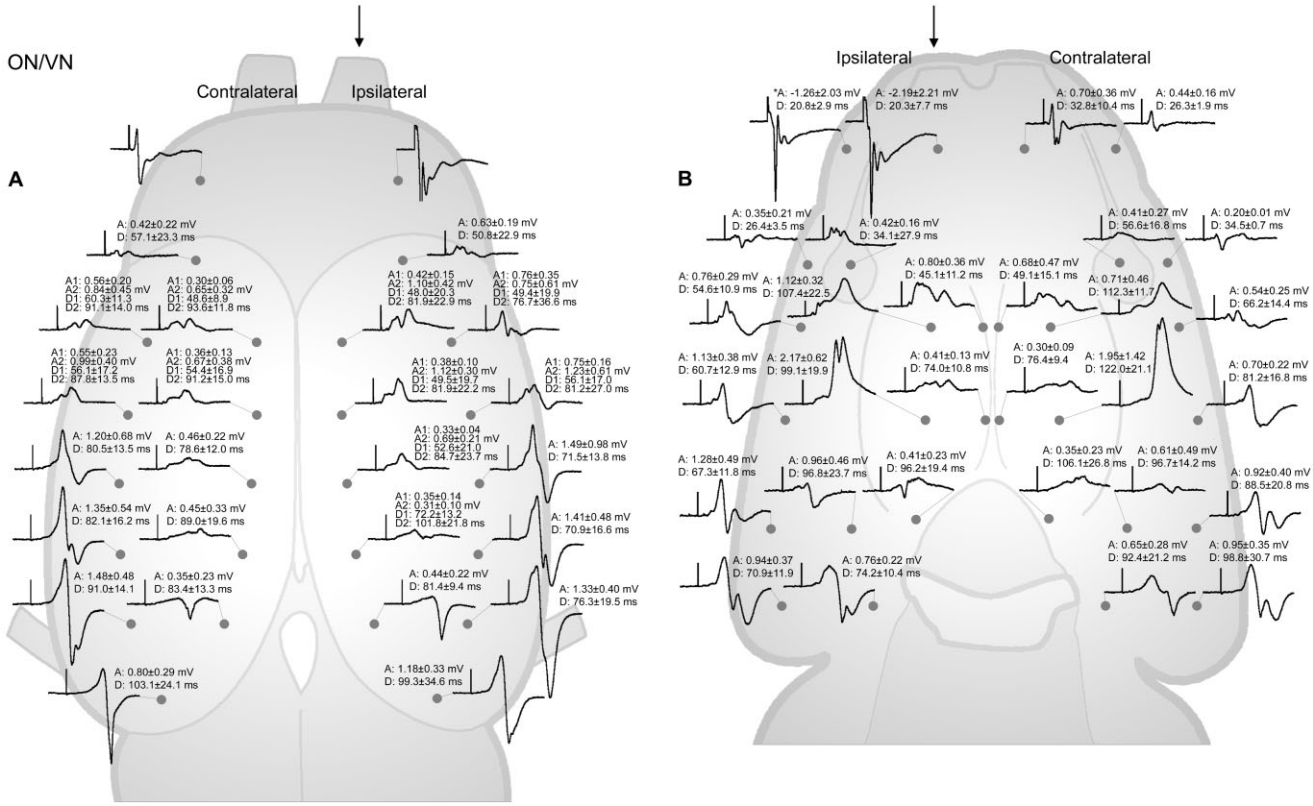


Fig. 4. Evoked potentials measured after stimulation of the olfactory/vomeronasal nerve in the fire-bellied toad. **A:** Dorsal view of the telencephalon showing responses to ipsilateral (right) and contralateral (left) stimulation of the olfactory/vomeronasal nerve. **B:** Ventral view of the telencephalon showing responses to ipsilateral (left) and contralateral (right) stimulation of the olfactory/vomeronasal nerve. Arrows show the location of the stimulating electrode. The traces shown were recorded in one animal and each trace represents an average of two responses. Recording sites are indicated on the schemata by gray dots. The values of response amplitude and delay to peak response for all animals are summarized above each trace (n = 4–5 in A and 2–5 in B). Evoked potentials at some pallial sites displayed two distinct peaks denoted 1 and 2. Values are means ± SD. Prestimulus time is 50 ms followed by a 0.7 ms stimulus and a 200 ms poststimulus period. The stimulus artifacts represent amplitude of 1 mV. The asterisk indicates that responses of both positive and negative polarity were recorded from that site.

conclusion can be made as to the region of potential reversal.

The use of additional stimuli generated evoked responses in the anterior medial pallium as shown in Figure 5. Responses were obtained after stimulation of the nerves 2Sp, IX-X-XI, VIII, and V. The response produced by stimulation of the obex is shown for comparison with the 2Sp response—two closely situated stimulation sites. The responses to stimulation of these nerves had long latencies, were often irregular from one stimulus to the other, and were mostly of low amplitude. Thus, the complete mapping of telencephalic responses was not pursued in these cases.

The effect of stimulation current intensity on the evoked responses was characterized by a quick increase in amplitude past threshold current to a maximum response, which would diminish significantly at high current values whether concentric or suction electrodes were used. Threshold current ranges were as follows: MO (0.04–0.2 mA), Torus (0.2–0.3 mA), OpN (0.02–0.15 mA), ON/VN (0.03–0.08 mA). Threshold stimulation currents for the additional stimulation sites were not studied in detail.

Dorsal thalamic evoked potentials

Evoked responses in the anterior medial pallium upon stimulation of the surface of the diencephalon varied depending on the site of stimulation (Fig. 6). Responses were reliably produced by stimulation of the anterior region above the habenula and dorsal thalamus. The responses were very similar to those obtained with ON/VN stimulation with two exceptions: 1) they did not habituate with short interstimulus intervals, and 2) they were comparatively minor in the main olfactory bulb (not shown). Note that sites 1 and 1′ in Figure 6 are located in the vicinity of the pretectum. The analysis of DT response characteristics surprisingly showed that the shortest latency of response was in the caudal lateral pallium and increased toward rostral sites (not shown). We considered thalamic afferents to the caudal lateral pallium unlikely (see below). In light of the many olfactory axons known to cross to the contralateral hemisphere in the habenular commissure, we suspected that antidromic activation of olfactory axons in the habenula was involved in the evoked potentials measured after DT stimulation. Therefore, the effect of LOT lesion on evoked responses generated by anterior

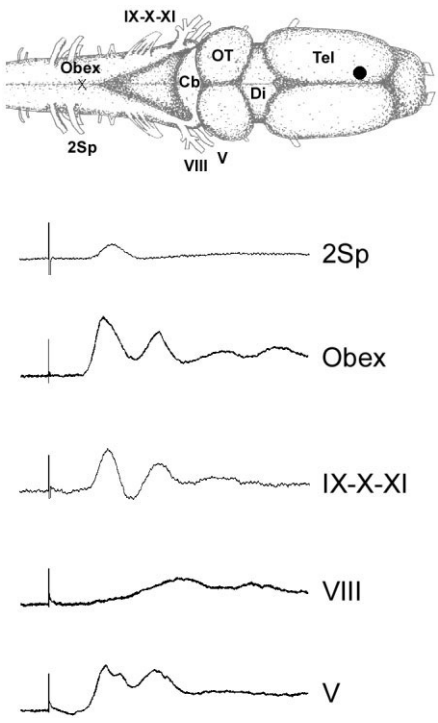


Fig. 5. More sensory-evoked potentials recorded in the medial pallium of the fire-bellied toad. Examples of evoked responses in the anterior medial pallium (black dot on the toad brain drawing) generated by stimulation of the contralateral dorsal horn of the second spinal nerve (2Sp), obex region of the caudal medulla oblongata, ipsilateral glossopharyngeal-vagal-accessory nerve bundle (IX-X-XI), contralateral octaval nerve (VIII), and contralateral trigeminal nerve (V). The traces shown were recorded in one animal and each trace represents an average of five responses. Prestimulus time is 50 ms followed by a 0.7 ms stimulus and a 450 ms poststimulus period. The stimulus artifacts represent amplitude of 0.5 mV. Cb, cerebellum; Di, diencephalon; OT, optic tectum; Tel, telencephalon.

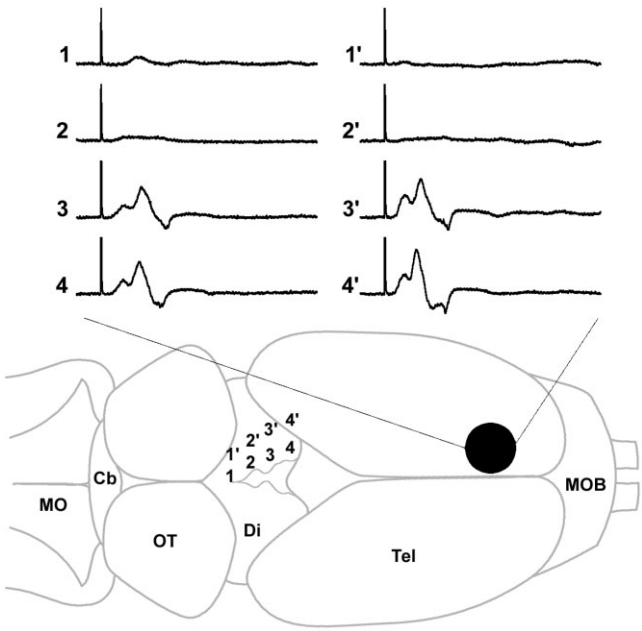


Fig. 6. Effect of variation of the dorsal thalamic stimulation site on evoked potentials in the medial pallium of the fire-bellied toad. Examples of evoked responses in the anterior medial pallium (black dot on the drawing of the toad brain) generated by stimulation of different sites in the dorsal thalamic region. Trace numbers are associated with their respective stimulation sites shown in the brain drawing below. The traces shown were recorded in one animal and each trace represents an average of five responses. Prestimulus time is 50 ms followed by a 0.7 ms stimulus and a 450 ms poststimulus period. The stimulus artifacts represent amplitude of 1 mV. Cb, cerebellum; Di, diencephalon; MO, medulla oblongata; MOB, main olfactory bulb; OT, optic tectum; Tel, telencephalon.

DT stimulation was investigated. Bilateral LOT lesion effectively abolished the second and largest peak of the prelesion evoked potential (Fig. 7A). This second peak persisted when LOT lesions were incomplete, albeit with longer delay and lower amplitude. In one animal the effect of an almost complete LOT lesion on the DT evoked potential is shown (traces above), whereas in another animal the second peak in response was completely eliminated due to a complete bilateral LOT lesion (traces below). Figure 7B shows an example of complete LOT lesion on a transverse brain section cut at the rostral preoptic level.

Evoked responses recorded in the dorsal striatopallidum after stimulation of the anterior DT were also similar to those obtained with ON/VN stimulation, but delayed by roughly 30 ms. The traces above in Figure 7C show the effect of LOT lesion on evoked responses in the dorsal striatopallidum after stimulation of the anterior DT. Responses generated by posterior DT stimulation were of negative polarity, as seen for MO and OpN-evoked potentials in the dorsal striatopallidum (see Figs. 1, 3), and were not affected by LOT lesion (Fig. 7C, traces below). For stimulation of the posterior DT, it was necessary to expose the medial surface of the diencephalon in longitudinally split brains. In that configuration, the responses in the medial pallium were of the same type as seen with stimulation of the anterior DT after LOT lesion in the intact brain (Fig. 7A). Thus, it appears that thalamic efferents to both the pallium and dorsal striatopallidum were simultaneously stimulated from a medial approach, and that the antidromic stimulation of the LOT was avoided.

Thalamotelencephalic pathways

Biocytin application to different structures of the anterior diencephalon revealed a variety of ascending pathways to the telencephalon, which are likely involved in the transmission of sensory afferents or the modulation of telencephalic responses. Due to the method of tracer application, only ipsilateral projections were studied. Biocytin was applied to the anterior nucleus of the dorsal thalamus (4; one case along with the thalamic eminence and 2 cases with a small part of the rostral central thalamus), the posterior thalamus (1), the habenula (2), and the thalamic eminence (1). Nomenclature of the diencephalon of *B. orientalis* in the present report is according to Roth et al. (2003) with additional contributions from Neary and Northcutt (1983) and Wicht and Himstedt (1988) regarding the identity of the thalamic eminence.

Anterior nucleus. One case of biocytin labeling restricted to the anterior nucleus of the dorsal thalamus is illustrated in Figure 8. It shows that a great quantity of

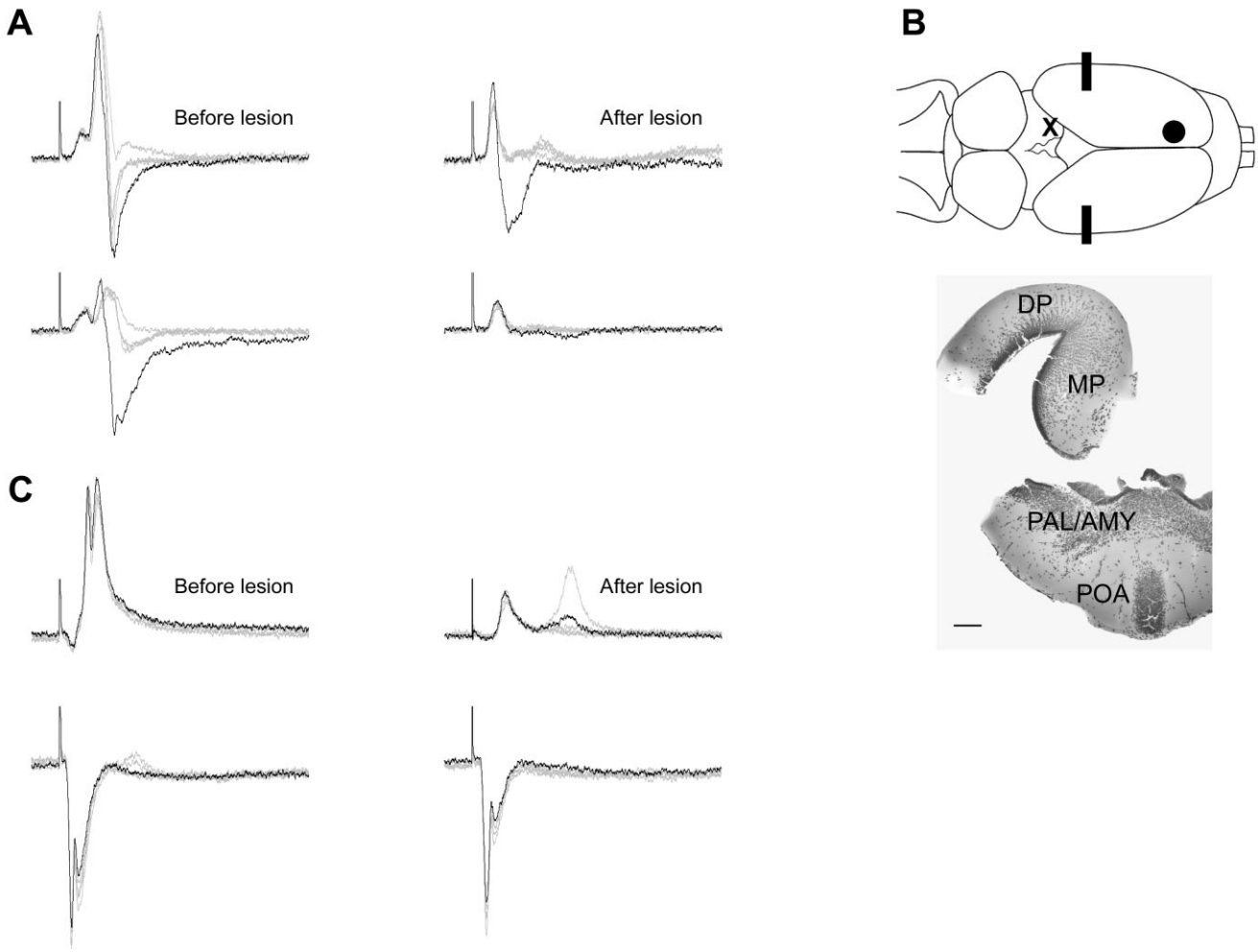
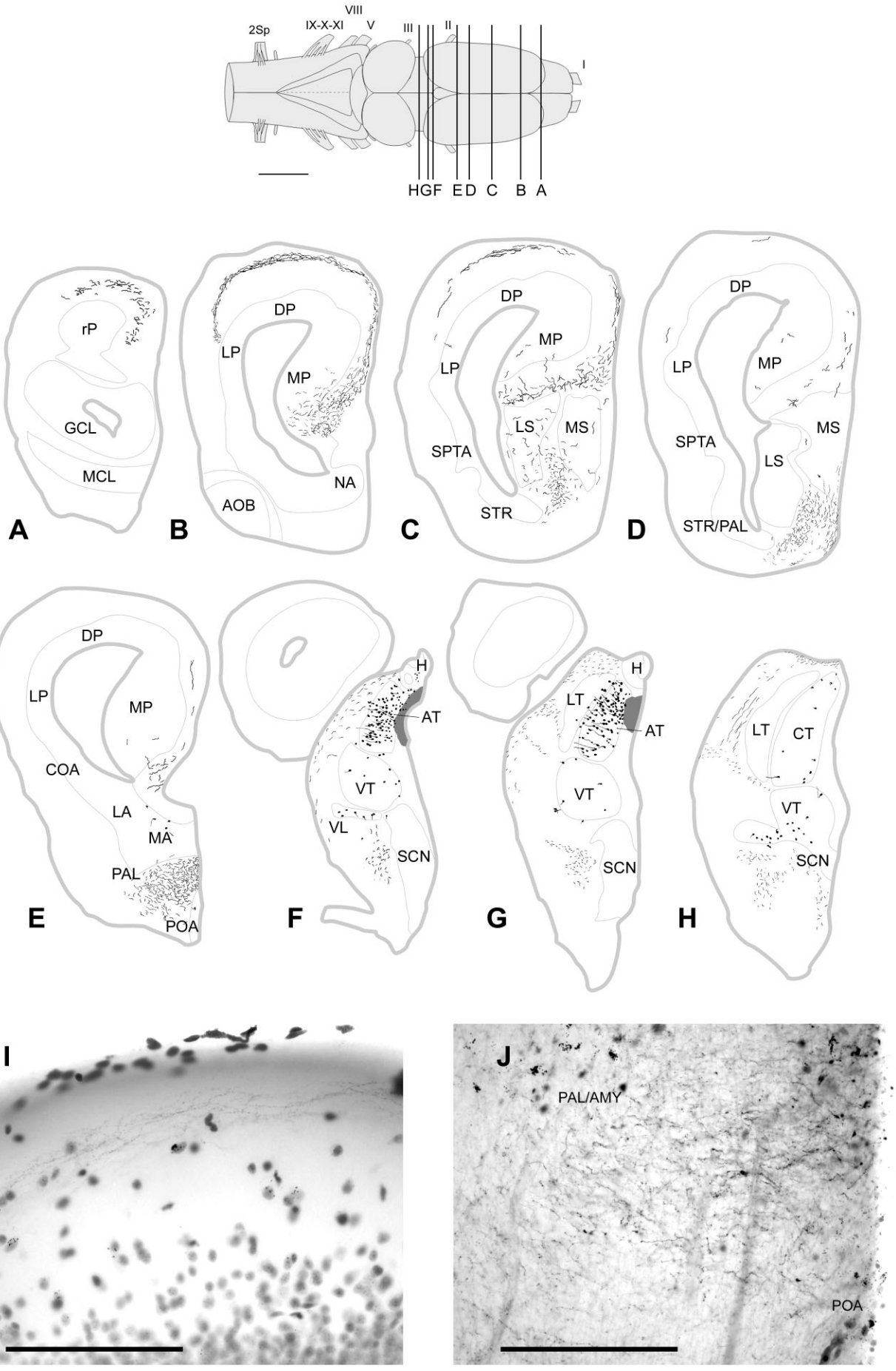


Fig. 7. Effect of bilateral lateral olfactory tract (LOT) lesion on evoked potentials generated by stimulation of the dorsal thalamus in the fire-bellied toad. **A:** Responses recorded in the anterior medial pallium after stimulation of the anterior dorsal thalamus before and after LOT lesion. Two examples from different animals are shown in the traces above and below, respectively. Each example shows five traces overdrawn. Prestimulus time is 50 ms followed by a 0.7 ms stimulus and a 450 ms poststimulus period. The stimulus artifacts represent amplitude of 1 mV. **B:** The stimulation and recording sites in A are illustrated on the brain drawing above by an X and a black dot, respectively, while LOT lesions are shown by black bars. Below is a photomicrograph of a transverse brain section showing a typical LOT lesion produced with fine scissors. Dorsal is upward. DP, dorsal pallium; MP, medial pallium; PAL/AMY, pallidum/amygdala region; POA, preoptic area. **C:** Responses recorded in the dorsal striatopallidum after stimulation of the anterior (traces above) or posterior (traces below) dorsal thalamus before and after LOT lesion. The brains were split longitudinally in order to expose the thalamic region to a medial approach. Time and amplitude scales are as in A. Scale bar = 500 μm in B.

neurons in the anterior nucleus took up the tracer. Ascending projections are strong in the medial region at the level of the amygdala (Fig. 8E,J) and weaker in the septum further rostrally. In all cases of biocytin application to the anterior nucleus, the major projection site, is the anterior region of the pallium (Fig. 8A,B), where varicose fibers extended in the white matter from the medial pallium to the lateral cellular prominence, which separates the dorsal and ventral parts of the lateral pallium. The fibers coursed within a thin band located progressively deeper within the white matter from the dorsal part of the medial pallium to the lateral pallium (Fig. 8I). Of note is the presence of labeled fibers up to the extreme rostral pole of the pallium above the main olfactory bulb. Less abundant fibers were seen in mid-rostrocaudal levels of the pallium (Fig. 8C), but were rarely seen outside the medial pallium at more caudal levels. Labeled fibers in the medial pallium were also notably less abundant at caudal levels. Retrogradely filled somata were observed in the ventrolateral thalamus and to a lesser extent in the central thalamus, subpallial amygdala, and medial septum. When the application sites included the rostral part of the central thalamus, an additional sparse projection to the dorsal striatopallidum was observed. In light of these findings and the results of Roth et al. (2003), which did not find anterior thalamic neurons projecting to the striatum using intracellular labeling in *Bombina*, it appears likely that the projection of the anuran anterior nucleus to the striatum reported by Neary (1990) was in fact a contribution of the rostral central thalamus. Additionally, none of


VIII
2Sp
IX-X-XI
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III
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HGF ED C B A
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GCL
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Figure 8

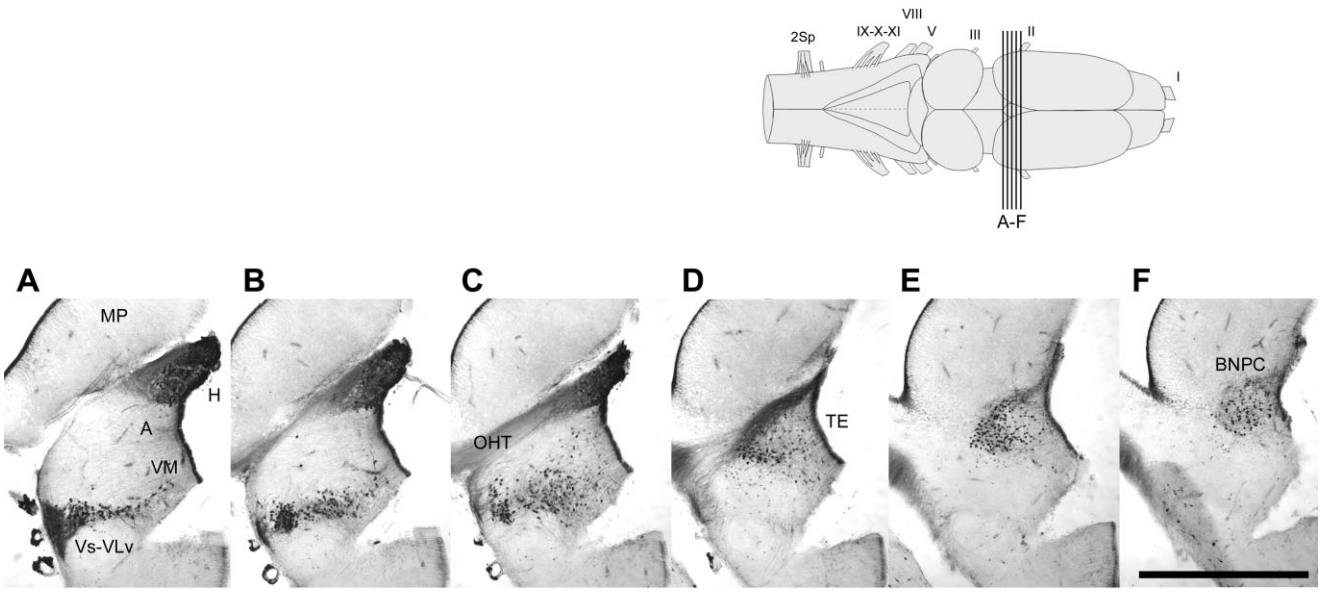

Fig. 9. Two major inputs to the habenula in the fire-bellied toad. **A–F:** A ventrolateral thalamic cell group and the thalamic eminence/bed nucleus of the pallial commissure are labeled after an application of biocytin crystals restricted to the habenula. The photomicrographs are successive transverse brain sections of 50 μm thickness. Dorsal is upward and lateral is to the left. Note that the ventromedial lesion seen in the brain was caused by postfixation damage. A, anterior nucleus of dorsal thalamus; BNPC, bed nucleus of the pallial commissure; H, habenula; MP, medial pallium; OHT, olfactohabenular tract; TE, thalamic eminence; VLv, ventral part of ventrolateral thalamic nucleus; VM, ventromedial thalamic nucleus; Vs, superficial ventral thalamic nucleus. Abbreviations in the schema above are as in Figure 8. Scale bar = 500 μm in F (applies to all).

the present cases of biocytin application to the anterior nucleus showed labeled fibers in the ventral portion of the lateral pallium or the main olfactory bulb as Neary (1990) reported.

Central nucleus. A strong projection to the whole rostrocaudal extent of the neuropil of the dorsal striatopallidum was observed only when biocytin was applied in the posterior dorsal thalamus, including the dorsal part of the ventral thalamus. This projection is well described and has been attributed to the central and lateral nuclei of the dorsal thalamus in anurans (Wilzcynski and Northcutt, 1983a; Marín et al., 1997a; Roth et al., 2003; Endepols et al., 2004).

Habenula. Two biocytin applications were nearly restricted to the habenula. In one case, the olfactohabenular tract was strongly backfilled and in the other case a small part of the caudal septum was involved in the application site in addition to the entire habenular region. Sparse ascending telencephalic projections to the medial septum and the ventral part of the medial pallium could be distinguished, but probably originated from backfilled cells in the bed nucleus of the pallial commissure (BNPC)/thalamic eminence (see below), since the habenula is not known to project to the septum or medial pallium in anurans (Northcutt and Ronan, 1992; Roden et al., 2005). The applications of biocytin to the habenula revealed important anatomical features of the diencephalon of *B. orientalis*, which are described in the following in order to establish a clear-cut definition of the thalamic eminence. The thalamic eminence is considered the most rostral diencephalic structure (Bergquist, 1932; Bradford and Northcutt, 1983; Puelles, 2001). In both cases of biocytin application to the habenula, retrogradely filled cells were observed in the septum, thalamic eminence, and BNPC, as well as in the ventrolateral and superficial ventral thalamic nuclei (Fig. 9). The thalamic eminence/BNPC appeared as a continuous structure situated rostral to the ventral thalamus and anterior nucleus in a position consistent with the position of the thalamic eminence described in a urodele amphibian (Wicht and Himstedt, 1988), but it is situated slightly more dorsal in *Bombina*. A strong projection of the thalamic eminence to the habe-

Fig. 8. Anatomy of the anterior thalamotelencephalic pathway in the fire-bellied toad. **A–H:** Labeling resulting from an application of biocytin crystals restricted to the anterior nucleus of the dorsal thalamus (dark gray in F,G). The images are camera lucida reconstructions of transverse brain sections cut at levels indicated on the above schema of the toad brain. Note the important biocytin uptake in the anterior nucleus of the dorsal thalamus. Projections posterior to the thalamus are not shown. **I:** Photomicrograph of a transverse brain section showing labeled thalamic fibers in the lateral part of the dorsal pallium (level B). **J:** Photomicrograph of a transverse brain section showing labeled thalamic fibers in the pallidum/medial amygdala region (level E). Dorsal is upward and lateral is to the left. I, olfactory nerve; II, optic nerve; III, oculomotor nerve; V, trigeminal nerve; VIII, octaval nerve; IX-X-XI, glossopharyngeal-vagal-accessory nerve bundle; 2Sp, second spinal nerve; AOB, accessory olfactory bulb; A, anterior thalamic nucleus; C, central thalamic nucleus; COA, cortical (main olfactory) amygdala; DP, dorsal pallium; GCL, granule cell layer of main olfactory bulb; H, habenula; L, lateral thalamic nucleus; LA, lateral (vomeronasal) amygdala; LP, lateral pallium; LS, lateral septum; MA, medial amygdala; MCL, mitral cell layer of main olfactory bulb; MP, medial pallium; MS, medial septum; NA, nucleus accumbens; PAL, pallidum; POA, preoptic area; rP, rostral pallium; SCN, suprachiasmatic nucleus; SPTA, striatopallial transition area; STR, striatum; VL, ventrolateral thalamus; VT, ventral thalamus. Scale bars = 500 μm in I,J.

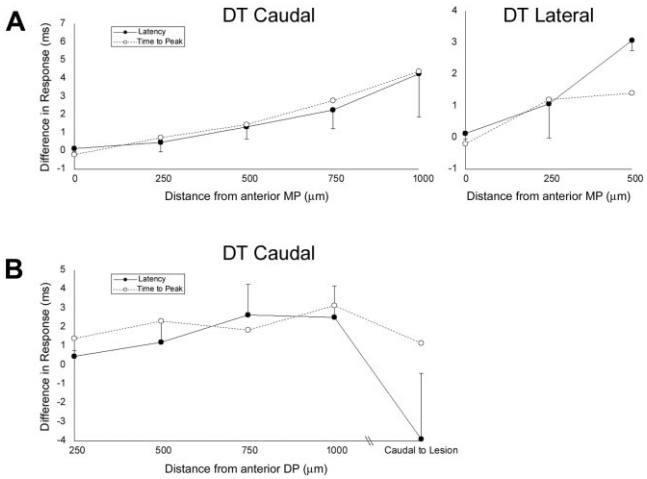

Fig. 10. Direction of spread of thalamic evoked potentials as revealed by simultaneous two-channel recording in the pallium of the fire-bellied toad. **A:** Upon stimulation of the dorsal thalamus the difference in response latencies increases as the distance caudal and lateral from the anterior medial pallium increases in toads with bilateral lateral olfactory tract lesion at caudal telencephalic levels. One electrode is moved away from the anterior medial pallium in caudal direction in the graph on the left, while it is moved in lateral direction in the graph on the right. **B:** The difference in response latencies increases slightly as the distance caudal from the anterior dorsal pallium increases, but the response at a site caudal to the olfactory tract lesion is recorded before the response in the anterior pallium (negative difference). Response latency is shown by filled circles and time to response peak is shown by open circles and broken line. Values are mean – SD (mean + SD in B) for latency and only mean for time to peak (n = 5). DP, dorsal pallium; DT, dorsal thalamus; MP, medial pallium.

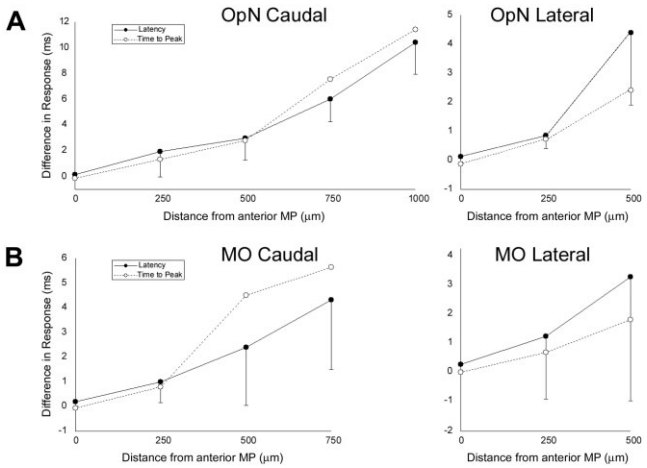

Fig. 11. Direction of spread of sensory evoked potentials as revealed by simultaneous two-channel recording in the pallium of the fire-bellied toad. **A:** Upon stimulation of the optic nerve the difference in response latencies increases as the distance caudal and lateral from the anterior medial pallium increases. One electrode is moved away from the anterior medial pallium in caudal direction in the graph on the left, while it is moved in lateral direction in the graph on the right. **B:** same experiment as in A, but the stimulus here is delivered to the medulla oblongata. Response latency is shown by filled circles and time to response peak is shown by open circles and broken line. Values are mean – SD for latency and only mean for time to peak (n = 5). MP, medial pallium; MO, medulla oblongata; OpN, optic nerve.

nula was also demonstrated in urodeles (Krug et al., 1993). The results in *B. orientalis* demonstrate that the thalamic eminence/BNPC extends from the pallial commissure to a caudal level previously described as the ventromedial thalamic nucleus in the bullfrog (Neary and Northcutt, 1983). Thus, the structure interpreted here as the thalamic eminence is larger and different from the structure identified under that name in Neary and Northcutt (1983), but fits with the topology and hodology of the thalamic eminence described in urodele amphibians (Wicht and Himstedt, 1988; Krug et al., 1993). The thalamic eminence/BNPC continuum as described here did not show a dorsoventral parcellation, but cells appeared smaller in the most rostral part, which we assume represents the BNPC, whereas the slightly larger cells in the caudal part are assumed to represent the thalamic eminence. As regards the other cell population backfilled in the habenula cases, the labeling in the ventrolateral thalamus was more abundant and extended more laterally in comparison to tracer applications involving the anterior nucleus. The migrated part of this cell population, which corresponds to the entopeduncular nucleus of Kemali et al. (1980) and the superficial ventral thalamic nucleus of Neary and Northcutt (1983), extended rostrally in a position lateral to the thalamic eminence (VM in Neary and Northcutt, 1983). In *B. orientalis*, it was not continuous with the anterior entopeduncular nucleus, which is situated rostral to level F shown in Figure 9.

Thalamic eminence/bed nucleus of the pallial commissure. A biocytin application restricted to the thalamic eminence/BNPC displayed sparse ascending telencephalic projections to the ventral part of the medial septum and the ventral medial pallium. A projection of the BNPC to the medial pallium in anurans was previously suggested from retrograde and anterograde tracing data (Kicliter, 1979; Northcutt and Ronan, 1992; Endepols et al., 2005). The reports of efferent connections of the BNPC in the anuran *Hyla versicolor* (Endepols et al., 2005) and the thalamic eminence in two urodele amphibians (Krug et al., 1993) show important similarities, which suggest that the same structure was investigated in both studies. The latter is made plausible by the seemingly coherent structure formed by the thalamic eminence/BNPC of *B. orientalis* observed in the present study. Intracellular labeling of neurons in the thalamic eminence of *B. orientalis* revealed that projections to the medial septum and medial pallium are more extensive than seen in the single case of biocytin application described above (G. Roth, unpubl. obs.).

Flow of evoked activity within the pallium

The analysis of response delay and amplitude in Figures 1–3 suggested that the MO, Torus, and OpN evoked potentials were generated in the anterior medial pallium. However, in these experiments the time between measurements made at two different positions depended on the recording sequence, and slight changes in response were sometimes observed with long intervals between measurements. In a further examination of the evoked potentials generated by DT, OpN, and MO stimulation, simultaneous two-electrode measurements were used to

TABLE 1. Summary of Sensory Responses Obtained by Intracellular Recording at Different Telencephalic Sites

	Brain Sites						
Response Type	rP	aMP	aDP	AMY	cPAL[1]	AMY/PAL	STR/DPAL
(+) OpN-MO	2 (1)[2]	3 (2)	5 (3)	1	1	(1)	2 (1)
(−) OpN-MO					1	(1)	3 (2)
(+) OpN-MO-DT	4 (1)						
(±) OpN-Torus			3 (3)				
(+) ON/VN-MO		1	1				4
(−) ON/VN-MO							3 (3)
(+) ON/VN-Torus		3 (2)		2		(1)	1
(−) ON/VN-Torus		3 (3)		1			2 (2)
(+) ON/VN-MO-Torus					1	(1)	
(+) ON/VN-MO-DT	1		1				1

[1]Includes anterior entopeduncular nucleus.
[2]Values in parentheses are the number of neurons recorded without or with unsuccessful biocytin injection.
Symbols: (+): excitatory response, (−): inhibitory response, (±): excitatory, then inhibitory response.
aDP, anterior dorsal pallium; aMP, anterior medial pallium; AMY, amygdala; AMY/PAL, amygdala/pallidum region; cPAL, caudal pallidum; DT, dorsal thalamus; MO, medulla oblongata; ON/VN, olfactory/vomeronasal nerve; OpN, optic nerve; rP, rostral pallium; STR/DPAL, dorsal striatopallidum; Torus, torus semicircularis.

ensure precise determination of response latency at pallial sites separated by increasing (or decreasing) distance. Furthermore, in a preceding experiment the mapping of DT-evoked potentials was shown to comprise olfactory antidromic activation, which prevented a clear-cut interpretation of the activity generated by DT stimuli. Thus, the additional two-electrode recording was essential to find out the direction of spread of thalamic activity inside the pallium. Figure 10A shows that the latency of DT response and the time to response peak both increase with distance from the anterior medial pallium in both caudal and lateral directions. The average speed of conduction was 0.345 mm/ms in the caudal direction and 0.163 mm/ms in the lateral direction. A similar effect was measured in the caudal direction in the dorsal pallium (Fig. 10B), which stands in contrast to the results described above when evoked responses were measured without LOT lesion. Figure 10B also shows that the response at a site caudal to the LOT lesion, where the course of olfactohabenular fibers was still not interrupted from the stimulation site, happened with a much lower latency. Figure 11 shows that OpN and MO-evoked potentials spread in the same directions as thalamic potentials. The average speed of conduction was 0.133 mm/ms (OpN) and 0.249 mm/ms (MO) in the caudal direction and 0.114 mm/ms (OpN) and 0.163 mm/ms (MO) in the lateral direction. The differences in conduction velocities ($n = 5$; measured between the anterior medial pallium and the farthest caudal location showing a clear evoked response in each animal) was statistically significant between DT and OpN stimulation (F-test > 0.05; $P = 0.004$ two-tailed Student's test for equal variances), but not significant between MO and OpN (F-test < 0.05; $P = 0.103$ two-tailed Student's test for unequal variances).

Intracellular responses

Table 1 lists the results of the intracellular recording experiment. In total, responses of 54 neurons were recorded with at least two types of sensory stimulation and 27 of them were successfully labeled by intracellular injection of biocytin. Figure 12A shows the location of the neurons labeled with biocytin. Biocytin labeling confirmed the regional boundaries deduced from visual observation of landmarks present on the surface of the dissected brain and identified the neuronal types recorded in the caudal pallidum/amygdala region (regional subdivisions as in Endepols et al., 2004; Laberge et al., 2006). In the latter region, a neuron was considered to belong to the ventral pallidum when the soma was located in a medial superficial position with dendrites oriented ventrolaterally reaching around the lateral forebrain bundle and with projections to the nucleus accumbens; neurons of the anterior entopeduncular nucleus (here considered a caudal part of the dorsal pallidum) had somata located in a lateral superficial position with dendrites oriented ventrolaterally and axonal projections to the dorsal striatopallidum, the pretectal/toral region and/or the optic tectum; somata of amygdala neurons were located deeper in the cellular layer with dendrites invading the ventromedial region of the subpallium (amygdala neurons responsive to sensory stimuli other than ON/VN were restricted to the medial subpallium). Representative examples of sensory responses recorded intracellularly are shown in Figure 12B–G. Intracellular recording revealed that all neurons found in telencephalic sensory regions of interest outside of the primary olfactory pallium (rostral/medial/dorsal pallium, amygdala/ventral pallidum, and dorsal striatopallidum) displayed bimodal sensory responses. Unimodal olfactory neurons were found in the lateral pallium (not shown). The responses were frequently excitatory, but inhibition or excitation followed by inhibition was also observed. For any given neuron, only one of the above response types was observed across stimulation sites. Dual intracellular and evoked potential recording showed that the evoked potential generally preceded intracellular potentials with DT, MO, Torus, and OpN stimulation. In the case of ON/VN stimulation, intracellular responses mostly overlapped with the course of the evoked potentials. It should be noted that ON/VN intracellular responses outside of the dorsal striatopallidum were often larger and persisted longer when compared with other types of stimulation. The all-or-none characteristics of MO responses in the amygdala exemplified in Figure 12F confirmed that orthodromic afferents were stimulated, as opposed to antidromic stimulation of fibers projecting to the MO originating from the subpallial amygdala.

Summation of sensory responses in the anterior medial pallium

Summation of sensory evoked potentials and intracellular responses was investigated in the anterior medial pallium for OpN-MO and ON/VN-MO pairs of stimuli. Figure 13A,B lists the values of evoked potential amplitude in mV for all animals tested (tables above the traces)

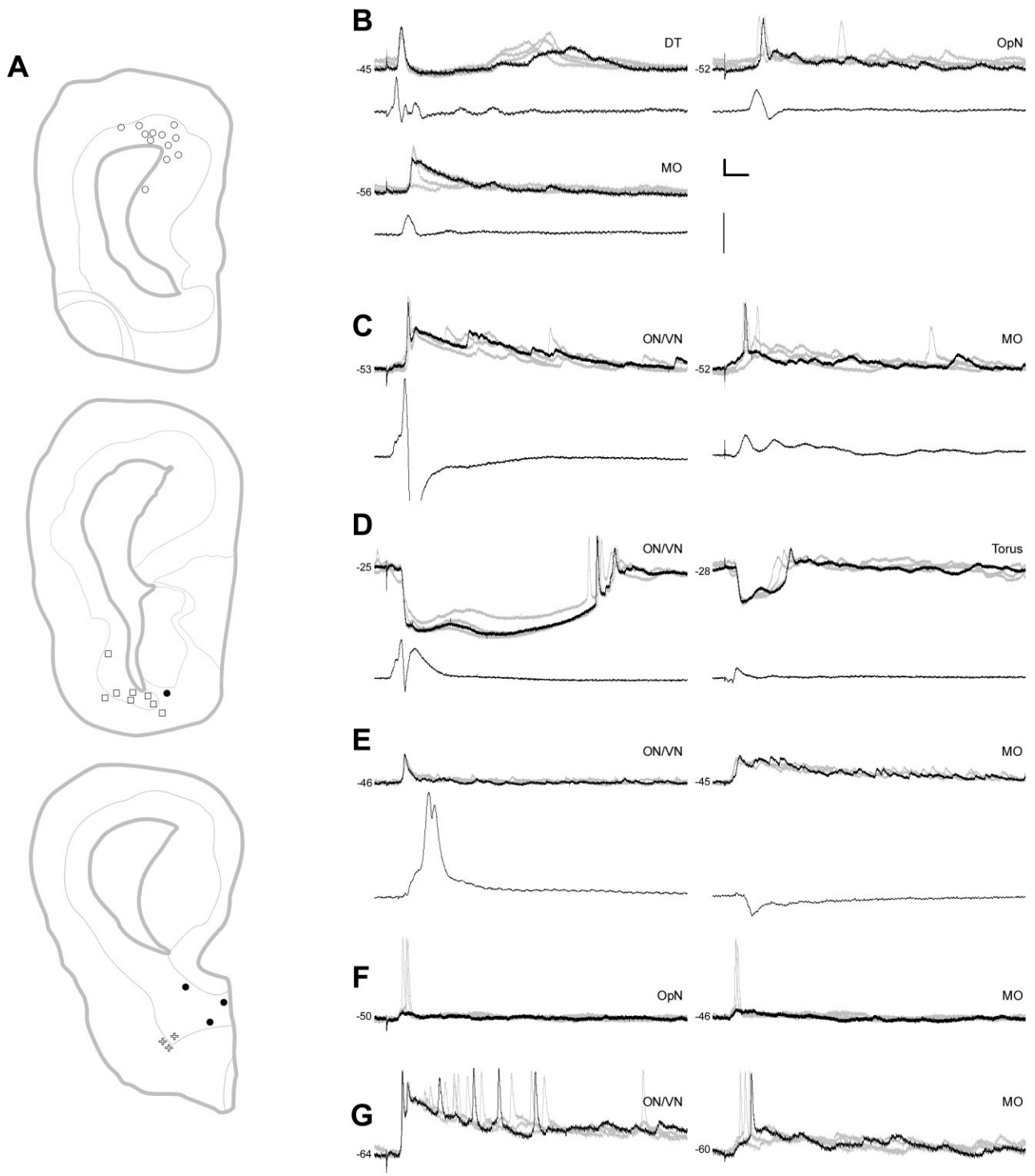

Fig. 12. Examples of sensory responses recorded intracellularly in telencephalic neurons in the fire-bellied toad. **A:** Location of all sensory neurons successfully labeled by intracellular injection of biocytin in the present study. Each symbol represents the soma location of one neuron or a cluster of two or three neurons obtained after a single injection. The somata of pallial neurons are represented by an open circle, amygdala neurons by a black circle, dorsal striatopallidum neurons by an open square, and caudal pallidal neurons by a cross. For ease of presentation, all somata are illustrated on the same side on the nearest neighboring of three rostrocaudal brain sections. See Figure 8 for the levels of section (B,D,E) and the identification of brain structures. **B–E:** Neurons situated in the rostral pallium (B), anterior medial pallium (C,D), and dorsal striatopallidum (E) show bimodal sensory responses (DT, dorsal thalamus; OpN, optic nerve; MO, medulla oblongata; ON/VN, olfactory/vomeronasal nerve; Torus, torus semicircularis). The intracellular responses above are four traces overdrawn, while the evoked potentials below are an average of four responses. Intracellular responses and evoked potentials were recorded simultaneously. B, below right: Vertical bars represent amplitude of 10 mV for intracellular records and amplitude of 1 mV for evoked potentials, respectively. The horizontal bar represents 100 ms. **F,G:** Neurons situated in the medial amygdala (F) and ventral pallidum (G) show bimodal sensory responses. The intracellular amplitude and time scales in B also apply to F,G. Prestimulus membrane potential (mV) is indicated on the left for each foreground (black) intracellular trace.

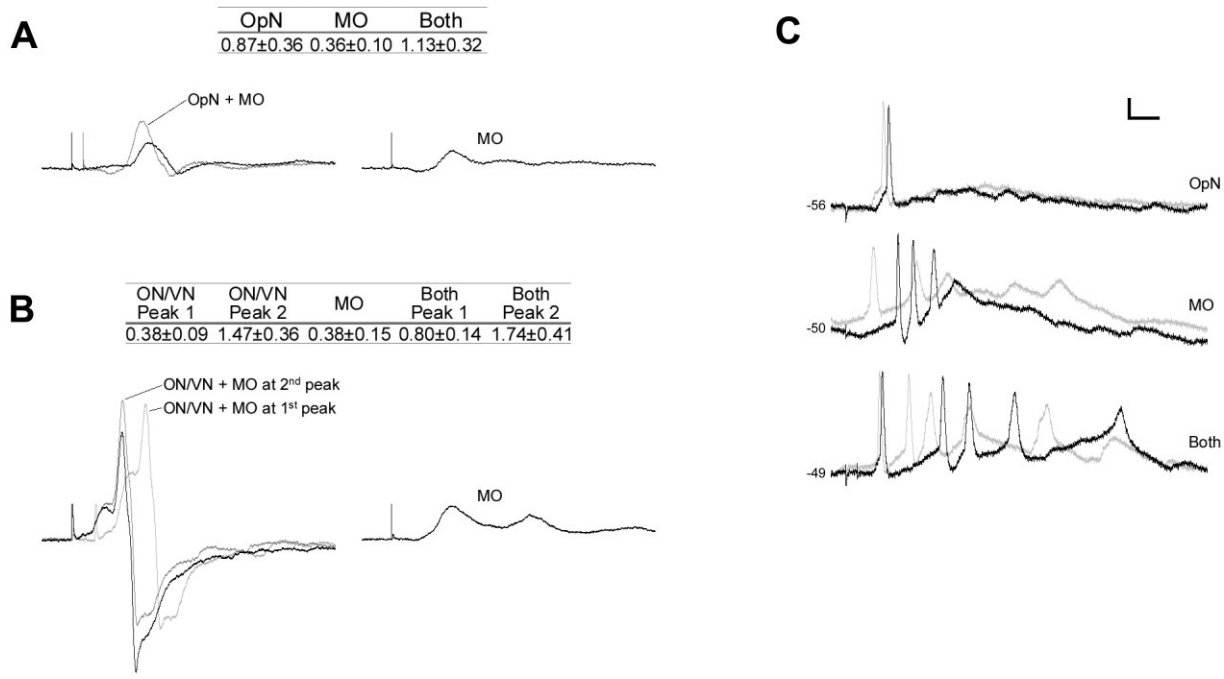

Fig. 13. Cross-modal summation of sensory responses in the anterior medial pallium of the fire-bellied toad. **A:** Evoked potential generated by stimulation of the optic nerve (black trace on the left) superimposed to the evoked potential generated by stimulation of both the optic nerve and the medulla oblongata (gray trace on the left). The dual stimulation was made so that the response peaks of both evoked potentials would coincide in time. The evoked potential generated by stimulation of the medulla oblongata alone is shown on the right. The traces shown were recorded in one animal and each trace is an average of four responses. Prestimulus time is 50 ms followed by a 0.7 ms stimulus and a 450 ms poststimulus period. The stimulus artifacts represent amplitude of 0.5 mV. The values of response amplitude (in mV) for all animals before and after summation are listed in the table above (n = 5). **B:** Evoked potential generated by stimulation of the olfactory/vomeronasal nerve (black trace on the left) superimposed to the evoked potential generated by stimulation of both the olfactory/vomeronasal nerve and the medulla oblongata (gray traces on the left) made to coincide with either the first or the second peak of the olfactory potential. The evoked potential generated by stimulation of the medulla oblongata alone is shown on the right. The traces shown were recorded in one animal and each trace is an average of three responses. Time and amplitude scales are as in A. The values of response amplitude (in mV) for all animals before and after summation are listed in the table above (n = 5, except for peak 1, where n = 3). **C:** Intracellular recording in a neuron of the rostral pallium showing responses produced by stimulation of the optic nerve, medulla oblongata, or both at the same time. In each case two traces are superimposed. The prestimulus membrane potential (mV) is indicated on the left for each foreground (black) intracellular trace. The vertical bar represents 10 mV and the horizontal bar 100 ms. MO, medulla oblongata; ON/VN, olfactory/vomeronasal nerve; OpN, optic nerve.

and examples of recordings taken from one animal. The results show that the amplitude of evoked potentials adds up precisely when the evoked responses triggered with both stimuli are made to coincide in time. An example of intracellular summation with OpN-MO stimuli in a medial pallial neuron is shown in Figure 13C. It shows that coincident stimulation of both sites appears to increase activity in comparison to separate stimulation of each site.

DISCUSSION

Sensory activity in the isolated *B. orientalis* brain

The sensory nature of the stimuli used in the present study is clearly established in the case of OpN, but stimulation of the ON/VN involved both main olfactory and vomeronasal afferents as well as a possible neuromodulatory influence of terminal nerve fibers (Wirsig-Wiechmann et al., 2002). Additionally, stimulation of the MO, Torus, and anterior DT regions were bound to simultaneously activate neighboring structures. In the case of MO stimuli, stimulation in the obex region likely activated the dorsal column nucleus (somatosensory) and the nucleus of the solitary tract (visceral and taste sensations), whereas stimulation in the central gray could have simultaneously activated the octavolateral area, superior olive, and more rostral parts of the dorsal column nucleus (acoustic-vestibular, acoustic, and somatosensory, respectively). In both cases the serotonergic median raphe was likely coincidentally stimulated. Stimulation of the toral region most likely involved somatosensory and auditory afferents, since these two modalities are known to be integrated in this structure (ten Donkelaar, 1998b). Thus, MO and Torus-evoked potentials were most likely composed of more than somatosensory afferents, which could explain the multiple responses peaks and following oscillations seen in the pallial responses. In the present study, stimulation of the anterior DT was shown to activate the olfactohabenular tract antidromically. However, this tract is not the only adjacent structure that could have been activated in that case; the habenula and thalamic eminence/BNPC were most likely activated since they are situated between the habenular tract and the anterior thalamic nucleus. The in vitro method used here had the

clear advantage of a reduced basal activity, which enabled the recording of responses with a practically silent baseline. This permitted the detection of low-amplitude responses and accurate measurement of response latency. However, the separation of each sensory modality, which is feasible in the intact animal, was not possible in vitro with the exception of the visual stimulus.

Evoked activity was shown to overlap across stimulation sites in three telencephalic regions: the anterior pallium, subpallial amygdala/ventral pallidum, and dorsal striatopallidum. Intracellular recordings suggest a multimodal nature of the neuronal elements in each of these regions. However, our protocol did not permit the definitive demonstration that all modalities target the same neurons, since each intracellularly recorded neuron could be stimulated from no more than two or three sites before the stimulating electrodes had to be moved. The result that additional nerve stimuli (2Sp, V, VIII, IX-X-XI bundle) always generated responses in the anterior medial pallium further supports the notion that this region is truly multimodal.

Evoked potentials appear to be the result of activation of axonal afferents since they consistently occurred before action potentials in dual evoked potential/intracellular recordings with the exception of olfactory evoked potentials, which persisted during action potential firing and sometimes displayed waves that correlated with the presence of action potentials.

Sensory afferents to the telencephalon are of three types: 1) unimodal olfactory afferents to the olfactory pallium and amygdala; 2) afferents from the anterior thalamic nucleus to the subpallial amygdala and anterior pallium that most likely represent integrated multimodal sensory information (Roth et al., 2003; Westhoff et al., 2004); and 3) afferents from the posterior dorsal thalamus to the dorsal striatopallidum. Nonolfactory responses in the amygdala/pallidum region were restricted to the subpallial (medial) amygdala, ventral pallidum, and anterior entopeduncular nucleus (here considered a caudal part of the dorsal pallidum). The latter observation argues against the existence of a functional equivalent of the multimodal mammalian basolateral complex in the pallial (lateral) parts of the amphibian amygdaloid complex, as proposed by Moreno and González (2003, 2004, 2006).

The results on response summation in the anterior medial pallium showed additive effects when different synapses (ON/VN-MO) as well as when modalities likely conveyed by the same anterior thalamic afferents (OpN-MO) were involved. In all cases intracellular responses were of the same polarity across stimulations sites. The latter suggests that summation of sensory information other than olfaction takes place in the thalamus or even earlier. Unfortunately, bimodal sensory responses of single neurons in the amphibian dorsal thalamus were found only in the posterior thalamus and not in the anterior thalamus (Vesselkin et al., 1971). Further studies in amphibians showed that anterior thalamic neurons projecting to the telencephalon display mostly inhibitory responses with long latencies after OpN stimulation (Roth and Grunwald, 2000; Roth et al., 2003). However, the few excitatory neurons in the anterior thalamus described in the latter studies showed habituation upon repetitive stimulation reminiscent of the sensory responses in the telencephalon reported here. It would be of interest to study if multimodal neurons and response summation are already present at the thalamic stage of sensory pathways.

Insights from the anatomy of afferent pathways to the telencephalon

Supin and Gusel'nikov (1965) suggested that pallial sensory potentials are generated in the medial pallium based on their observation that response amplitude is greater there. The present study shows that the shortest latencies of pallial responses to MO, Torus, OpN, and DT correlate with the extent of projections from the anterior dorsal thalamic nucleus and that the flow of responses within the pallium proceeds in rostral to caudal and medial to lateral directions. The previous notion that evoked activity can spread passively across brain regions (Supin and Gusel'nikov, 1965; Northcutt et al., 2004) is not supported by the present study. Our results show a clear delineation of fastest response latencies in regions receiving sensory thalamic input, but no or only delayed activity in the caudal pallium, despite the fact that the latter is situated closer to the stimulation sites or the sensory relay stations in the tectum, torus, and thalamus where sources of early sensory activity are situated.

The higher intrapallial conduction velocities from the site of origin of DT responses compared to OpN responses came as a surprise. Conduction velocity of MO responses also appeared to be increased in comparison to OpN, but this tendency was not significant, probably due to the small sample size. The above observation also argues against passive electrotonic flow of evoked responses as a major component of response conduction velocity measured in the pallium and suggests that modulation of conduction velocity can occur through a general effect on ion channels of pallial neurons and/or at synapses mediating intrapallial connections. The anatomical substrate for intrapallial transmission is very rich in amphibians, where pallial pyramidal neurons display extensive axonal networks and a high abundance of varicosities throughout the pallium. Suspected structures that project to the pallium and could have been simultaneously stimulated in the DT and MO cases are the BNPC/thalamic eminence and the serotonergic median raphe, respectively. The BNPC/thalamic eminence telencephalic projections are limited to the septum and medial pallium (Kicliter, 1979; Krug et al., 1993; Endepols et al., 2005; present study), but they spread extensively at these sites as shown by intracellular labeling (G. Roth, unpubl. obs.). Fibers originating from that region do not course in the medial forebrain bundle, as is the case for the anterior thalamic efferents, but they run dorsally in the ventral medial pallium/caudal septum region and innervate these structures from caudal to rostral. Thus, it is unlikely that they transmit sensory input because sensory responses correlate with the pallial projection field of the anterior thalamic nucleus as described above. Diffuse serotonergic afferents from the raphe to the medial and dorsal pallia have been described in amphibians (Parent, 1975; Dicke et al., 1997; Mühlenbrock-Lenter et al., 2005). Other neuromodulators known to abundantly innervate the pallium in *Bombina* are neuropeptide Y, somatostatin, and substance P (Mühlenbrock-Lenter et al., 2005). Unfortunately, the neurochemistry of projections of the BNPC/thalamic eminence to the medial pallium is not known. The BNPC/thalamic eminence could modulate pallial activity through a putative projection to the basal forebrain

cholinergic neurons in the septum, which are known to make up a pathway targeting the ventral part of the medial pallium in amphibians (González and Lopez, 2002; Sánchez-Camacho et al., 2006). Alternatively, most likely complementarily, the similar connection between the subpallial amygdala and the septal region in *B. orientalis* could be involved in the above-mentioned modulation of pallial activity, as suggested by results in the rat showing that amygdala input to the cholinergic basal forebrain enhances isocortical and hippocampal activity (Dringenberg and Vanderwolf, 1996). Experiments involving pharmacological alterations are needed to substantiate the present speculation that sensory pallial activity can be modulated by nonsensory afferents in amphibians. Interestingly, cholinergic input to the mammalian cortex has been shown to enhance the processing of thalamic input and to increase the size of sensory representations in the cortex (Sarter et al., 2005), and was implicated in the modulatory effects of attention on cognition (Sarter et al., 2006). Of further interest is that an activating role for serotonergic projections to the cortex has also been shown in mammals, and it was suggested that direct cholinergic and serotonergic inputs are essential for cortical activation and mediate the effects of all other systems involved in cortical activation (Dringenberg and Vanderwolf, 1998).

The axons of neurons of the central thalamic nucleus are known to arborize extensively along the rostrocaudal extent of the dorsal striatopallidum neuropil (Roth et al., 2003). Analogous responses in rostral and caudal parts of the dorsal striatopallidum apparently reflect this common thalamic input. However, evoked responses of different polarities were obtained according to whether they were mediated by the thalamus or the olfactory pathway. The negative evoked responses recorded after stimulation of the MO, Torus, OpN, and posterior DT contrast sharply with the large positive olfactory potentials. A possible reason for this striking difference could be that the thalamic and olfactory sensory afferents target different dendritic compartments in the dorsal striatopallidum. Such a compartmentalization of afferents to the dorsal striatopallidum has been described in salamander, where projections of the olfactory amygdala restricted to the outer neuropil are present (Laberge and Roth, 2005). It is also reflected by a clear compartmentalized expression of some neuropeptides and transmitters as seen by immunohistological staining (Mühlenbrock-Lenter et al., 2005). A similar phenomenon is observed in the very rostral part of the pallium, where nonolfactory evoked potentials are of negative polarity, contrasting with the positive polarity of the evoked responses in the caudally adjacent pallial regions. The reason for this difference is unclear, but neurons in the rostral pallium display atypical dendrites that are oriented rostrally (Roth et al., 2007). An alternative explanation could be that the anterior thalamic afferents target a more superficial zone of the white matter in the rostralmost pallium in comparison with the more caudal regions.

On the role of the amphibian telencephalon

In anurans, direct olfactory input targets the accumbens/septum/rostral medial pallium region through the medial olfactory tract of the olfactory bulb and the whole extent of the lateral pallium, the lateral part of dorsal pallium, and the cortical amygdala through the lateral olfactory tract (Northcutt and Royce, 1975; Scalia et al., 1991; Roth et al., 2004). The present study showed that olfactory/vomeronasal nerve stimulation generates activity at every telencephalic site. The largest olfactory responses were measured in the caudal pole of the telencephalon, which confirms its role as a primary olfactory region, as was suggested from its strong input from the main olfactory bulb (Roth et al., 2004). The topology of the latter region is equivalent to the lateral entorhinal cortex of early mammals (Voogd et al., 1998; Martinez-Marcos and Halpern, 2006). Olfactory activity is strong and persistent in comparison to other sensory modalities in the telencephalic sensory regions outside of the dorsal striatopallidum, where it appears equal to the other modalities. Overall, these findings suggest a strong olfactory influence on telencephalic functions. The reason for this widespread olfactory activity appears to be the presence of many, and possibly exclusively, multimodal regions besides the primary olfactory telencephalon. The telencephalic sensory regions in *B. orientalis* are thus the primary olfactory pallial structures and a number of multimodal regions in the pallium, subpallial amygdala, ventral pallidum, and dorsal striatopallidum. The large and slow olfactory responses recorded in the dorsal striatopallidum are unlikely to be the result of direct olfactory input, which if present is limited to a small rostrodorsal part in this region (Marín et al., 1997a). Candidate structures relaying olfactory information to this region are the telencephalic structures that project to the dorsal striatopallidum and receive direct ON/VN input; namely, the vomeronasal and main olfactory amygdala (AL in Wilczynski and Northcutt, 1983a; MEA and LA in Moreno and González 2003, 2004), and possibly the dorsolateral rostral pallium (see below). Another tentative candidate region is the SPTA/VP (striatopallial transition area/ventral pallium), in which neurons display axonal varicosities in the outer neuropil of the dorsal striatopallidum on their way to the ventral telencephalon, and might receive both main and accessory olfactory bulb input.

The biological importance of multimodal sensory integration in amphibians is highlighted by recent studies demonstrating that the release of aggressive behavior in the dart-poison frog depends on bimodal visual and auditory cues presented in a coherent temporal and spatial fashion (Narins et al., 2003, 2005). Similarly, prey-catching behavior in salamanders is improved by the combination of visual and olfactory cues (Roth, 1976; Lindquist and Bachmann, 1982; Luthardt and Roth, 1983). The latter two phenomena show that both species-specific and learned responses make use of multimodal integration in amphibians. How could individual multimodal neurons participate in these behaviors? The observed summation of multimodal sensory inputs in the anterior medial pallium of *B. orientalis* is one candidate mechanism. Studies in mammals have identified two regions of convergence of multimodal sensory input in the non-isocortical pallial telencephalon: the limbic prefrontal and anterior cingulate cortices (reviewed in Calvert, 2001). Interestingly, functional imaging in humans demonstrated that these brain regions showed greater activity when bimodal stimuli were presented in a coherent fashion, as opposed to when each stimulus conveyed a different meaning (Laurienti et al., 2003). Thus, the basic neural mechanisms that extract behaviorally meaningful information from multimodal sensory signals could also be present in the amphibian pallium. Mechanisms of multi-

modal integration are better studied with natural stimuli that can be varied in intensity, timing, and location, as shown by the properties of multimodal collicular neurons in the cat (Kadunce et al., 1997; Stanford et al., 2005). The studies of Narins et al. (2003, 2005) confirm that amphibians could make good models to study the neural mechanisms of multimodal integration.

The few functional studies of the amphibian pallium are of great interest here. Lesion of the rostral telencephalon impaired the habituation of goal-directed behavior in a salamander (Pietsch and Schneider, 1990). More targeted studies showed that lesion of the medial pallium in anurans strongly attenuate the habituation of orienting behavior with persistent presentation of prey-like stimuli, as well as the habituation of learned instrumental responses during extinction (Finkenstädt and Ewert, 1988; Muzio et al., 1993, 1994). Lesion of the medial pallium also prevented adjustment to changes in reward magnitude in toads (Papini et al., 1995). Importantly, these lesions had no effect on the acquisition of instrumental learning. Note that the latter authors used large lesions, which are likely to interrupt thalamic projections to the anterior pallium. Another study used salamanders trained to raise their heads at presentation of a large rectangle stimulus in order to get a reward and investigated the effect of diverse lesions in the pallium (Wenz and Himstedt, 1990). Lesions of the medial or dorsal pallia had little effect on the conditioned response. Unfortunately, the animals were not tested for extinction. Surprisingly, lesions of the lateral pallium abolished the conditioned response completely and the animals could not relearn the task with further training. It is important to note here that the lesions the authors identified as covering the lateral pallium also included the dorsal part of the dorsal striatopallidum (see their fig. 2). The importance of the latter finding will be discussed below. The effects of pallial lesions observed in amphibians are reminiscent of the effects of lesions of the limbic mediodorsal thalamus (Corbit and Balleine, 2003) and prefrontal cortical structures on instrumental behavior in mammals (Ferry et al., 2000; Killcross and Coutureau, 2003; Dalley et al., 2004), as well as serotonin depletion in the prefrontal region (Clarke et al., 2005). This is in agreement with the well-established role for structures of the mammalian prefrontal cortex in inhibition of inappropriate responding.

What are the roles of the two sensory afferent systems to the amygdala/pallium and the dorsal striatopallidum in amphibians? The balance of the evidence suggests that the anterior pallium is involved in the modulation of goal-directed behavior, with increased attention associated with novelty and error detection in action-outcome playing a role, whereas the learning of a task is established elsewhere in the brain. The dorsal striatopallidum could be involved in habit formation and thus represent the site where a learned task is established. As important supporting evidence of the latter, intracellular labeling of neurons in the dorsolateral part of the rostral pallium in *Bombina* showed abundant projections to the nucleus accumbens and an additional projection restricted to the dorsal part of the dorsal striatum (Roth et al., 2007). The latter projection is one of the few direct pallial pathways to the dorsal striatopallidum in anurans; other projections are from the olfactory amygdala, possibly the SPTA/VP targeting the outer striatal neuropil, and a small ill-defined projection from the ventral part of the medial pallium (Neary, 1990; Marín et al., 1997a; Westhoff and Roth, 2002; Roth et al., 2007). Interestingly, as mentioned above, lesions involving the dorsal part of the dorsal striatopallidum in an amphibian abolished a learned response as well as the relearning of that response with extensive training (Wenz and Himstedt, 1990). Thus, the situation in amphibians strongly resembles the mammalian prefrontal circuits involved in the behavioral sequence of goal-dependent performance followed by habit formation, which are respectively attributed to the prefrontal cortex and dorsal striatum (Yin et al., 2004, 2005, 2006; Everitt and Robbins, 2005). Regions of anatomical convergence between the outputs of the pallium and dorsal striatopallidum in amphibians are found in the ventral thalamus (only medial pallium) and dorsal hypothalamus (Wilczynski and Northcutt, 1983b; Neary, 1990; Marín et al., 1997b; Westhoff and Roth, 2002).

The subpallial amygdala, which receives the same sensory input from the anterior thalamic nucleus as the anterior pallium in *B. orientalis* (Roth et al., 2003), participates in still undefined ways in the attribution of motivational and affective significance to stimuli (Balleine and Killcross, 2006). The amphibian brain also displays abundant possibilities for regulation of network responses, as exemplified by reciprocal connections (feedback loops) between regions. The pallium displays such reciprocal connections with the septum, subpallial amygdala, anterior dorsal thalamus, and thalamic eminence (Neary, 1990; Westhoff and Roth, 2002; Roth et al., 2004). The dorsal striatopallidum has reciprocal connections with the ventral (weak) and posterior thalamus, posterior tuberculum, and isthmus (Wilczynski and Northcutt, 1983a,b; Marín et al., 1997a,b). Clearly, despite the presence of fewer brain regions in comparison to amniotes, we are still in the early stages of figuring out how the brains of amphibians function as whole systems.

In conclusion, the amphibian telencephalon without any doubt plays an important role in multimodal sensory associations. The presence of many telencephalic anatomical subdivisions that process multimodal sensory input in series or in parallel combined with the results of functional studies in amphibians suggest that these networks display functional subdivisions involved in different aspects of emotional, motivational, and adaptive behavior. The present results in *B. orientalis* suggest the involvement of the amphibian pallium in the extraction of behaviorally meaningful information from multimodal sensory signals and its likely modulation by attention. The dorsal striatopallidum could be involved in habit formation by converting dorsolateral rostral pallial input into sensorimotor memories. There are important similarities between amphibians and mammals regarding the limbic pathways involved in the regulation of motivated behavior.

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