Properties of Conditioned Abducens Nerve Responses in a Highly Reduced In Vitro Brain Stem Preparation From the Turtle

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Anderson, Curtis W. and Joyce Keifer. Properties of conditioned abducens nerve responses in a highly reduced in vitro brain stem preparation from the turtle. *J. Neurophysiol.* 81: 1242–1250, 1999. Previous work suggested that the cerebellum and red nucleus are not necessary for the acquisition, extinction, and reacquisition of the in vitro classically conditioned abducens nerve response in the turtle. These findings are extended in the present study by obtaining conditioned responses (CRs) in preparations that received a partial ablation of the brain stem circuitry. In addition to removing all tissue rostral to and including the midbrain and cerebellum, a transection was made just caudal to the emergence of the IXth nerve. Such ablations result in a 4-mm-thick section of brain stem tissue that functionally eliminates the sustained component of the unconditioned response (UR) while leaving only a phasic component. We refer to this region of brain stem tissue caudal to the IXth nerve as the “caudal premotor blink region.” Neural discharge was recorded from the abducens nerve following a single shock unconditioned stimulus (US) applied to the ipsilateral trigeminal nerve. When the US was paired with a conditioned stimulus (CS) applied to the posterior eighth, or auditory, nerve using a delay conditioning paradigm, a positive slope of CR acquisition was recorded in the abducens nerve, and CR extinction was recorded when the stimuli were alternated. Resumption of paired stimuli resulted in reacquisition. Quantitative analysis of the CRs in preparations in which the caudal premotor blink region had been removed and those with cerebellar/red nucleus lesions showed that both types of preparations had abnormally short latency CR onsets compared with preparations in which these regions were intact. Preparations with brain stem transections had significantly earlier CR offsets as more CRs terminated as short bursts when compared with intact or cerebellar lesioned preparations. These data suggest that a highly reduced in vitro brain stem preparation from the turtle can be classically conditioned. Furthermore, the caudal brain stem is not a site of acquisition in this reduced preparation, but it contributes to the sustained activity of both the UR and CR. Finally, the unusually short CR onset latencies following lesions to the cerebellum are not further exacerbated by removal of the caudal brain stem. These studies suggest that convergence of CS and US synaptic inputs onto the abducens nerve reflex circuitry may underlie acquisition in this reduced preparation, but that mechanisms that control learned CR timing arise from the cerebellorubral system.

INTRODUCTION

Studies suggest that more than one neuronal circuit underlies the generation of the reflexive eyelid/nictitating membrane response in vertebrates. This behavior is generated by the cooperative action of nearly all of the extraocular muscles (Berthier 1992; Evinger and Manning 1993). During eye blinks in rabbits, the orbicularis oculi muscle closes the eyelid while the retractor bulbi and other extracocular muscles retract the orbit, which causes the nictitating membrane to passively sweep across the eye. Thus, on the efferent side, several cranial nerve motoneuron pools may potentially be involved in the generation of eye blinks. Stimulation of the trigeminal nerve, which supplies the afferent innervation of the orbit, produces two distinct components of the blink reflex in a variety of mammalian species that have been studied; the early R1 and late R2 responses (Berthier and Moore 1983; Evinger et al. 1988; Hiraoka and Shimamura 1977; Pelligrini et al. 1995). Recent studies have shown that R1 and R2 are two independent responses mediated by distinct and parallel neuronal pathways (Pellegrini et al. 1995; Van Ham and Yeo 1996). In the guinea pig and rabbit, the short-latency R1 component is generated by several divisions of the trigeminal nucleus. The longer latency R2 response is generated by circuitry originating in the C1 region of the spinal cord. Given these findings, two main questions arise. First, how is the activity of these independent circuits integrated to generate the coordinated behavior that is observed? Second, which (or both) of these distinct reflex responses and their associated neuronal pathways undergoes modification during classical conditioning of the eyelid/nictitating membrane response? The latter question was addressed in the present study by eliminating part of the circuit that generates an R2-like component of a neural correlate of the eye blink response in an in vitro preparation from the turtle. This preparation was examined for its capacity to be classically conditioned.

The present study utilizes an in vitro model of classical conditioning in the turtle. Stimulation of the trigeminal nerve produces discharge recorded in the ipsilateral abducens nerve that resembles electromyographs (EMGs) of extraocular muscles recorded during blinks. This discharge is considered to be a neural correlate of the eye blink reflex in the turtle (Keifer 1993). When trigeminal nerve stimulation (the unconditioned stimulus, US) is paired with stimulation of the auditory nerve (the conditioned stimulus, CS), neural discharge in the abducens nerve can be recorded in response to the CS that shows a positive slope of acquisition as pairing proceeds, and extinction in response to unpaired stimuli (Anderson and Keifer 1997; Keifer et al. 1995). Lesion studies using this preparation have further shown that robust acquisition of abducens nerve conditioned responses (CRs) can be obtained in the absence of the red nucleus and the entire cerebellum (Anderson and Keifer...
Here, we extend these findings by obtaining CRs in preparations that received a partial ablation of the brain stem circuitry. In addition to removing all tissue rostral to and including the midbrain and cerebellum, a transection was made just caudal to the emergence of the glossopharyngeal (IX) nerve. Such an ablation results in an ~4-mm thick section of brain stem tissue that contains afferent and efferent nerves, and a portion of the abducens reflex circuitry. This transection functionally eliminates the late, sustained component of the unconditioned response (UR) while leaving only the early phasic component (similar to R2 and R1 of mammals, respectively) (Keifer 1993). The data suggest that this highly reduced in vitro brain stem preparation from the turtle can be classically conditioned. Furthermore, the integrity of the sustained component of the UR, i.e., the R2-like component, is not required to obtain the abducens nerve CRs recorded here. Thus the causal brain stem is not a site of CR acquisition in this reduced preparation, but it normally contributes to the sustained activity of both the UR and CR. Finally, the unusually short onset latencies of the CR following removal of the cerebellum (Anderson and Keifer 1997) are not further exacerbated by removal of the caudal brain stem. These studies suggest that direct convergence of synaptic inputs from the CS and US onto the abducens nerve reflex circuitry (Herrick and Keifer 1998) may underlie acquisition in this reduced preparation, but that mechanisms that control learned CR timing arise from the cerebellorubral system. This work has been presented in abstract form (Anderson and Keifer 1998).

METHODS

In vitro preparation

The preparation of the isolated turtle brain stem–cerebellum has been described previously (Keifer 1996). Pond turtles (Chrysemys picta; n = 25; carapace length of 10–15 cm) were anesthetized by hypothermia and decapitated (Marcus 1981; Parsons and Huggins 1965). The brain and upper cervical spinal cord were dissected out and the preparation was transferred to the recording chamber where it was continuously bathed in physiological saline containing (in mM) 100 NaCl, 6 KCl, 40 NaHCO₃, 2.6 CaCl₂, 1.6 MgCl₂, and 20 glucose, oxygenated with 95% O₂–5% CO₂ and maintained at room temperature (22–24°C) at pH 7.6. The telencephalon was transected and discarded, and the dura covering the preparation was carefully dissected away. The brain stem was transected at the level of the cerebellar peduncles were transected to remove the entire cerebellum and the trochlear nerve to remove tissue containing the red nucleus, and the preparation was transferred to the recording chamber where it was continuously bathed in physiological saline containing (in mM) 100 NaCl, 6 KCl, 40 NaHCO₃, 2.6 CaCl₂, 1.6 MgCl₂, and 20 glucose, oxygenated with 95% O₂–5% CO₂ and maintained at room temperature (22–24°C) at pH 7.6. The telencephalon was transected and discarded, and the dura covering the preparation was carefully dissected away. The brain stem was transected at the level of the trochlear nerve to remove tissue containing the red nucleus, and the cerebellar peduncles were transected to remove the entire cerebellum including the deep cerebellar nuclei. After abducens nerve reflex responses were recorded, an additional transection was made just caudal to the emergence of the IXth nerve.

Stimulation and recording of abducens nerve responses

Fire-polished glass suction electrodes were used for stimulation and recording of the cranial nerve rootlets. Neuronal discharge was recorded in the abducens nerve following single shock stimulation (0.1 ms duration, usually about 2 times threshold) of the ipsilateral trigeminal nerve trunk. The abducens nerve projects to the retractor bulbi, lateral rectus, and pyramidalis muscles that control the turtle eye (Barbas-Henry and Lohman 1986, 1988). The retractor bulbi retroretracts the globe of the eye into the orbit, passively raising the nictitating membrane, whereas the pyramidalis actively moves the nictitating membrane and eyelid (Duke-Elder 1958; Walls 1942). The lateral rectus muscle abducts the orbit. This abducens nerve discharge is considered to be a neural correlate of the eye-blink reflex (Keifer 1993) and served as the UR in the conditioning experiments. After initial recordings were made from the abducens nerve following trigeminal nerve stimulation, the brain stem was transected just caudal to the level of the IXth nerve root. This transection functionally eliminates the sustained component of the UR while leaving only the phasic component (see RESULTS). Extracellular signals were amplified with a band-pass of 10 Hz to 3 kHz, recorded on videocassette tape (Vetter), and reproduced on a chart recorder (Astro-Med).

Conditioning protocol

The procedures for conditioning the in vitro turtle brain stem preparation have been described previously (Anderson and Keifer 1997; Keifer et al. 1995). A delayed-conditioning protocol in which the CS immediately precedes the US was used. The CS consisted of a 100-Hz, 1-s train stimulus (0.1 ms duration pulses) applied to the posterior root to the eighth nerve. This nerve has been shown to contain primarily auditory and some vestibular fibers (Foster and Hall 1978; Herrick and Keifer 1998). The stimulus amplitude of the CS ranged from 58 to 82% (72 ± 2.3%, mean ± SE) of the threshold current that produced activity in the abducens nerve. The CS immediately preceded a single shock US applied to the ipsilateral trigeminal nerve. The intertrial interval was 30 s. Each pairing session consisted of 50 CS-US stimulus trials followed by a 30-min rest period in which there was no stimulation. If the preparation showed reliable CRs, alternate-pairing sessions in which the CS and US were separated by 10 s were used to test for CR extinction and possible sensitization or pseudoconditioning effects. The alternate pairing was continued until the CR showed extinction, and then the paired CS-US trials were resumed to test for reacquisition of the response.

Data analysis

A CR was defined as neuronal discharge recorded in the abducens nerve that occurred during the CS and had an amplitude of at least 25% of the unconditioned response (Anderson and Keifer 1997; Keifer et al. 1995). The total number of CRs per block of 10 stimuli and per session (5 blocks per session) were recorded. The latency of the onset of the CR was measured as the time period from the onset of the CS to the initiation of the CR. The latency of the offset of the CR was measured as the time period from the onset of the CS to the offset of the CR. In addition, the amplitude was measured by integrating the spike density of the CR using the public domain image analysis program NIH Image (available at http://rsb.info.nih.gov/nih-image/). Using this program, the number of pixels that comprise the CRs from the different preparations were measured. These data were converted to percentages with respect to the intact preparations, which were defined as 100% amplitude. Paired-sample t-tests, regression analyses, and univariate ANOVAs were done on a PowerMac using StatView statistical software (Abacus Concepts, Calabasas, CA). Comparisons of the slopes of the regression equations for Fig. 5B were performed with a t-test using the standard error of the difference between the regression coefficients. Statistical significance was set at P < 0.05. Acquisition, extinction, and reacquisition rates were calculated as the mean number of CRs divided by the number of sessions. Data are presented as means ± SE.

Histology

Following the conditioning procedures, the preparations were examined histologically to determine the extent of the lesions. At the end of the experiment, the tissue was immersion fixed in 4% paraformaldehyde and sectioned at 60 μm using a sliding microtome. Sections were stained with thionin to facilitate assessment of the rostrocaudal
RESULTS

Twenty-five in vitro brain stem preparations from the turtle *Chrysemys picta* were used in this study. Of these 25 preparations, 13 (or 52%) exhibited a positive slope of acquisition of CRs. In the results, we will first describe the transections of the caudal brain stem and the resultant effect on the UR recorded from the abducens nerve. This will be followed by a description of the conditioning of the abducens nerve response and the characteristics of the CRs in these brain stem preparations. The present data will be compared with previous studies of classical conditioning of the turtle in vitro abducens nerve response that used preparations in which the cerebellum and red nucleus were intact (Keifer et al. 1995) and those in which the cerebellum and red nucleus were removed (Anderson and Keifer 1997).

Properties of the UR following brain stem transections near nerve IX

All of the in vitro brain stem preparations reported here first received transections that removed tissue rostral to and including the midbrain and cerebellum (transection *a* in Fig. 1D). Removal of these tissues has no effect on the generation of the trigeminally evoked eye blink response recorded from the abducens nerve (Keifer 1993) (Fig. 1A). This reflex response is characterized as having two components; an initial short-duration synchronous discharge having a duration of ~50–150 ms, and a longer, sustained component that has a duration of up to several seconds (Keifer 1993). Dual short- and long-duration components of the trigeminally evoked eye blink reflex have been reported in all mammalian species studied (Berthier and Moore 1983; Evinger et al. 1988; Hiraoka and Shimamura 1977; Pelligrini et al. 1995). Once these URs were recorded, the brain stem was then transected at the level of, or just caudal to, the glossopharyngeal nerve (transection *b* in Fig. 1D).

![Diagram](http://jn.physiology.org/Download/1244.jpg)
TABE 1. Summary of CR properties of preparations in which the cerebellum, red nucleus, and caudal brain stem have been removed

<table>
<thead>
<tr>
<th>Case</th>
<th>Tissue Past nIX, µm</th>
<th>CR Onset, ms</th>
<th>CR Offset, ms</th>
<th>CRs/Session, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1,200</td>
<td>153 ± 5</td>
<td>240 ± 7</td>
<td>21</td>
</tr>
<tr>
<td>2</td>
<td>3,250</td>
<td>43 ± 1</td>
<td>57 ± 17</td>
<td>9</td>
</tr>
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<td>3</td>
<td>2,250</td>
<td>159 ± 22</td>
<td>256 ± 54</td>
<td>22</td>
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<tr>
<td>4</td>
<td>840</td>
<td>211 ± 10</td>
<td>529 ± 45</td>
<td>19</td>
</tr>
<tr>
<td>5</td>
<td>120</td>
<td>292 ± 20</td>
<td>634 ± 32</td>
<td>15</td>
</tr>
<tr>
<td>6</td>
<td>840</td>
<td>268 ± 23</td>
<td>743 ± 34</td>
<td>8</td>
</tr>
<tr>
<td>7</td>
<td>720</td>
<td>398 ± 35</td>
<td>586 ± 50</td>
<td>15</td>
</tr>
<tr>
<td>8</td>
<td>1,020</td>
<td>252 ± 20</td>
<td>573 ± 44</td>
<td>12</td>
</tr>
<tr>
<td>9</td>
<td>840</td>
<td>237 ± 5</td>
<td>541 ± 44</td>
<td>9</td>
</tr>
</tbody>
</table>

Data are from 9 preparations that showed CR acquisition, extinction, and reacquisition. Tissue past nIX indicates the amount of brain stem that remained intact past the emergence of the IXth nerve following the transection. CR onset is the mean latency (±SE) of CR onset with respect to the beginning of the CS; CR offset is the mean latency (±SE) of CR offset with respect to the beginning of the CS. The rate of acquisition is indicated as CRs/session and is the mean percent of CRs per session during the acquisition phase of the experiment.

Previous work (Keifer 1993) has shown that this transection abolishes the sustained component of the reflex while leaving the phasic response unaffected, and this was confirmed in the present study. Figure 1B shows representative recordings from the abducens nerve in which only the short-duration component of the trigeminally evoked response remained after removal of the caudal brain stem. The inset in Fig. 1B is of a recording at a faster time scale showing that the response is ~50 ms in duration. Quantitative measurements of the duration of these reflex responses following transection of the caudal brain stem demonstrate that they were significantly shorter than were responses recorded before removal of this tissue (Fig. 1C; mean ± SE, prelesion data 3,780 ± 587 ms, postlesion data 132 ± 27 ms; F = 38.6; P < 0.0001). Experiments in which a series of consecutive transections from caudal to rostral were made suggest that the region contributing to the long-duration component of the reflex is between the vagus (X) and IXth nerves, near the level of the obex.

The placement of the transections was examined histologically to determine the extent of tissue that remained following these extensive lesions. The initial transections removed tissue rostral to the level of the trochlear nerve and cutting the cerebellar peduncles resulted in the removal of the entire cerebellum. The transections of the caudal brain stem ranged from 120 to 3,250 µm (1,168 ± 288 µm) past the emergence of the IXth nerve (Table 1). All of these transections were rostral to the vagus nerve and resulted in complete elimination of the long-duration component of the UR. This final transection resulted in a thick section of brain stem tissue that ranged from 2,460 to 5,400 µm (3,625 ± 249 µm) in total rostrocaudal extent. This dramatically reduced brain stem preparation that generates only the short-duration component of the UR was examined for its capacity to be classically conditioned.

Conditioning following removal of caudal brain stem

Paired stimuli were applied to the posterior eighth nerve and the ipsilateral trigeminal nerve to examine whether this reduced brain stem preparation could exhibit classically conditioned abducens nerve responses. Data from one case (case 9, Table 1) that exhibited acquisition, extinction, and reacquisition of the abducens nerve response is shown in Fig. 2. Figure 2A shows the degree of CR acquisition plotted as the percent of CRs during the training sessions. During the first three pairing...
sessions, the percentage of CRs increased ~9% of CRs per session to a total of 28% CRs by the third acquisition session. The training protocol was then changed to unpaired stimuli consisting of alternate CS-US trials to examine whether the preparation would show extinction. Two sessions of unpaired stimuli were applied during which the number of CRs declined to 12%. Paired CS-US trials were resumed, and CRs were reacquired at a faster rate of 27% per session, and a greater number of CRs were expressed (66% CRs by the end of session 7). Figure 2B shows the same data as in Fig. 2A, but they are plotted in 5 blocks of 10 stimuli per session to show greater resolution of the acquisition curve. Examples of extracellular abducens nerve recordings are shown during pairing sessions that produced a CR (a, arrow), early extinction trials that produced a CR (b, arrow), extinction trials in which no CR was recorded (c), and during reacquisition of the CRs (d, arrow).

Data from another experiment (case 2) that demonstrated a positive slope of acquisition followed by extinction are shown in Fig. 3. During the first three pairing sessions, the percentage of CRs increased at a rate of ~9% of CRs per session to a total of 26% CRs by the third session. Two sessions of alternate pairing followed during which the number of CRs extinguished to a total of 8% by the end of the fifth session. Presentation of paired stimuli was resumed, and as in the case described above, reacquisition at a faster rate of 15% of CRs per session to a total of 38% was recorded in sessions 6 and 7.

Of the 25 preparations that were tested, 13 exhibited acquisition of conditioned responses. However, 4 of these 13 were excluded from further analysis due to either the presence of spontaneous activity that prevented quantification of the data, or because the preparation failed to generate a complete acquisition, extinction, and reacquisition curve. An analysis of grouped data from the nine remaining preparations (Table 1) showed no significant differences between any of the cases for the latency of CR onset ($F = 15.88; P = 0.06$), the latency of CR offset ($F = 4.32; P = 0.20$), the total number of CRs recorded during the experiment ($F = 0.49; P = 0.79$), or the rate of CR acquisition per session ($F = 0.70; P = 0.69$). Furthermore, there were no significant interactions between any of these measurements and the total amount of tissue or the amount of tissue past nerve IX that remained intact. Therefore data from all nine cases were combined to generate the histogram shown in Fig. 4. During the initial pairing sessions, the number of CRs increased at a mean acquisition rate of 15% CRs per session to a mean of 44% CRs by the third pairing session. The preparations were then presented with alternate CS and US stimuli to test for extinction. The three sessions of extinction stimuli resulted in a mean decrease of CRs at a rate of 13% per session to a mean of 4% CRs by the final extinction session. Pairing was resumed, and reacquisition of CRs occurred at a faster rate of 20% per session as compared with the initial period of acquisition. By the second reacquisition session, a mean of 30% CRs was observed.

The acquisition, extinction, and reacquisition of abducens nerve CRs observed in the present study suggests that it is possible to classically condition an in vitro turtle brain stem preparation following removal of tissue posterior to nerve IX. However, a number of features of these CRs differed when they were compared with CRs obtained from preparations having a fully intact cerebellum/red nucleus and those in which the cerebellum and red nucleus had been removed.

![Figure 3](http://jn.physiology.org/)

**Figure 3.** Acquisition curves from a different brain stem preparation (case 2). A: 3 pairing sessions resulted in CR acquisition to 26%. Two extinction sessions of alternate CS-US followed, and the percent of CRs declined to 8%. Reacquisition to 38% occurred after resuming the CS-US pairing. B: same data as in A, but divided into 5 blocks of 10 stimuli plotted for each session.

**Characteristics of CRs in brain stem preparations**

Several parameters of the CRs were analyzed including the latency of onset, latency of offset, and amplitude. Figure 5A shows the mean latency of the CR onset for intact cerebellum/red nucleus preparations (data from Keifer et al. 1995), those in which the cerebellum/red nucleus were removed (data from Anderson and Keifer 1997), and from the nine cases presented here that underwent an additional transection of the caudal brain stem. The latency of the CR onset, defined as the time from the beginning of the CS to the beginning of the CR, did not differ between the preparations from this study and those in which the cerebellum and red nucleus were removed ($F = 0.27; P = 0.613$). However, both of these groups had CR...
onsets that were significantly shorter when compared with the intact preparations ($F = 29.25; P < 0.0001$). The intact preparations had a mean CR onset of 392 ± 51 ms. This was significantly longer than the cerebellum/red nucleus lesioned preparations (mean of 242 ± 8 ms, $P < 0.005$) or the preparations that additionally received a transection of the caudal brain stem (mean of 219 ± 36 ms, $P < 0.01$). In addition, the latency of CR onset typically shifts to later periods during the CS as training proceeds. The latency of CR onset as a function of the training session for all preparations is shown in Fig. 5B. The slope of the shift in CR onset latency during training for the preparations in which the caudal brain stem had been transected (Δ) did not differ from that observed in preparations in which the cerebellum and red nucleus had been removed (●; Fig. 5B; $t = 0.77; P = 0.47$). However, the shift in the CR onset latency of both of these groups was significantly attenuated when compared with the intact cerebellum/red nucleus preparations (○; $t = 3.14; P < 0.05$). These data suggest that the timing mechanisms that play a role in the shift of the CR onset over the course of training in intact preparations are significantly affected by removal of the cerebellum, but are not additionally altered by transection of the caudal brain stem.

The offset of the CR is defined as the time from the initiation of the CS to the termination of the CR. Figure 6A shows the differences in CR offset among intact preparations, those in which the cerebellum/red nucleus were removed, and in those preparations that additionally received a transection of the caudal brain stem. There were significant differences between all three treatment groups for the CR offset ($F = 29.26; P < 0.0001$). For the intact preparations, the mean CR offset was 980 ± 7 ms, indicating that the CRs were usually sustained and lasted to the occurrence of the US. For those preparations in which the cerebellum and red nucleus had been removed, the mean CR offset was 794 ± 26 ms ($P < 0.01$ as compared with intact preparations). The cases that underwent transection of the cerebellum/red nucleus and the caudal brain stem demonstrated CRs that were significantly shorter than the other two treatment groups and had a mean offset of 462 ± 75 ms ($P < 0.0001$ as compared with either the intact or Cb/RN lesioned groups). These CRs were nearly always short bursts (as shown in Fig. 2 and in the bottom panel of Fig. 7A) and terminated before the onset of the US.

In addition to the timing characteristics that differed among the treatment groups, the amplitude of the CR was significantly smaller than CRs measured from either the intact preparations or those with cerebellum/red nucleus lesions (Fig. 6B).
CRs from intact preparations were compared with those that received cerebellum/red nucleus lesions, there were no significant differences in the amplitude of the CR (Fig. 6B; 100 ± 12% vs. 102 ± 15%, respectively; F = 0.07; P = 0.94). However, the preparations in which the caudal brain stem had been transected generated CRs that had a significantly reduced amplitude (47 ± 34%; F = 6.91; P < 0.05). This is largely due to the fact that CRs were not sustained. Figure 7A shows examples of extracellular recordings of typical abducens nerve CRs generated from a preparation with a cerebellum/red nucleus lesion and from a preparation that underwent the additional removal of the caudal brain stem. Conditioned responses measured from preparations in which the red nucleus/cerebellum had been removed were usually sustained to the initiation of the US (Anderson and Keifer 1997). Thus the CR offset occurred on average earlier in the CS than did that of the intact preparations. Preparations with the caudal brain stem transection had significantly shorter duration and smaller amplitude CRs than those recorded from either of the other two types of preparations, and they typically appeared as burst responses.

DISCUSSION

Taken together, the results of the present study, those from Keifer et al. (1995), and Anderson and Keifer (1997) have implications for identifying regions in the turtle brain that contribute to different properties of the CR such as acquisition and timing. In the first part of the DISCUSSION, the contribution of the caudal brain stem to abducens nerve eye blink responses will be described. Second, comparisons will be made of the CRs obtained from the different types of in vitro preparations from the turtle that have been tested and the implications of these findings. Last, potential mechanisms for CR acquisition in this reduced brain stem tissue will be discussed in light of recent neuroanatomic data that describe the CS and US nerve projections in this species.

Caudal premotor blink region

The abducens nerve eye blink reflex circuitry of the turtle, like mammals, is distributed in the brain stem, and the different components of the blink response appear to be mediated by distinct neuronal pathways. Removal of tissue containing the...
cerebellum and red nucleus has no effect on an in vitro trigeminally evoked neural correlate of the eye blink response recorded from the abducens nerve. However, transection of caudal regions of the brain stem at the level of the glossopharyngeal nerve results in the elimination of the late, sustained component of the response while leaving a monosynaptic, phasic discharge. Brain stem transections suggest that the late, sustained component of the turtle blink response is likely to be generated, at least in part, by neurons located between the levels of the glossopharyngeal and vagus nerves, near the obex. This region will be referred to as the “caudal premotor blink region.”

The early and late components of the abducens nerve eye blink response in the turtle may be analogous to the R1 and R2 components of blinks recorded in all mammalian species that have been studied (Berthier and Moore 1983; Evinger et al. 1988; Hiraoka and Shimamura 1977; Pelligrini et al. 1995). The neuronal pathways that underlie early and late eyelid movements in mammals have been studied in detail. Pelligrini et al. (1995) found that hemisections in the C1 region of the spinal cord abolish the R2 component of trigeminally evoked blink responses recorded in the orbicularis oculi muscle of the guinea pig. Activity of this muscle results in eyelid closure and is controlled by the facial nucleus. Hemisections at the level of the obex result in elimination of the R1 response. Tract tracing studies in the guinea pig have confirmed projections from the C1 region of the spinal cord and caudal parts of the trigeminal nuclei to the orbicularis motoneurons (Pelligrini et al. 1995). Similar findings have been described in the rabbit (Van Ham and Yeo 1996). The neuronal circuits underlying the turtle eyelid/nictitating membrane responses are somewhat different in that transections that eliminate the R2-like component are near the level of the obex, farther rostral than in the guinea pig, and transections just posterior to the eighth nerve do not eliminate the R1-like component. Thus the circuitry under study in the turtle is more rostrally located than in the guinea pig; however, these circuits control the abducens motoneurons rather than the facial nucleus, which may explain this difference. The motor component of the facial nucleus in reptiles is involved primarily in the function of the muscles of mastication (Székely and Matezs 1993), and, because turtles lack muscles of facial expression, it may not have a role in eye blinks. Findings from an anatomic study of the rabbit corneal-abducens nerve reflex are consistent with the physiological results reported here. Injections of horseradish peroxidase into the brain stem 2 mm caudal to the abducens nucleus resulted in anterograde label in the abducens and trigeminal motoneurons, whereas injections 4–5 mm caudal resulted in label in the facial and hypoglossal motoneurons (Harvey et al. 1984). Thus circuits controlling abducens nerve reflexes appear to be more rostrally located in the pontine and medullary reticular formation (Gacek 1979; Harvey et al. 1984; Weiss and Disterhoft 1985) than those controlling facial or hypoglossal reflexes.

In the turtle, the caudal premotor blink region contains several nuclear groups that could serve as premotor interneurons in these pathways. These nuclei include the raphe, the inferior reticular formation, the descending vestibular nucleus, and the nucleus of the descending spinal trigeminal tract (Cruce and Nieuwenhuys 1974). Injections of neurobiotin into the trigeminal nerve of the turtle reveal axons of the spinal trigeminal tract that descend into the spinal cord. Labeled neurons, likely by transneuronal transport, have been observed near the level of the obex. These neurons may be involved in generating the sustained component of the abducens nerve blink response, but these pathways remain to be described.

**Comparison of CR properties in different in vitro preparations**

The major finding of the present study is that this highly reduced brain stem preparation can be classically conditioned. Acquisition of CRs is obtained when preparations are presented with paired stimuli and extinction of CRs occurs in response to alternate stimuli (Figs. 2–4). However, the CRs are severely attenuated. In a previous study of the in vitro conditioned abducens nerve response, CRs recorded from preparations in which the cerebellum/red nucleus had been removed had significantly shorter onset latencies as compared with intact preparations (Anderson and Keifer 1997). These results are similar to those reported by Perret et al. (1993), who found that short-latency CRs were produced following lesions of the cerebellar cortex in the rabbit. Moreover, in the turtle, the shift in the CR onset latency as training proceeded was significantly attenuated when preparations with cerebellar lesions were compared with those that were intact. The present study extended these findings by obtaining CRs in in vitro preparations that had a transection of the caudal brain stem in addition to complete removal of the cerebellum and red nucleus. The latency of CR onset does not differ between brain stem transected preparations and those with cerebellum/red nucleus lesions, but both of these groups have significantly shorter latency CR onsets as compared with the intact preparations (Fig. 5A). Moreover, the latency of CR offset in brain stem preparations occurs significantly earlier during the CS than that recorded in either the intact or cerebellar lesioned preparations (Fig. 6A). The CRs generated by the brain stem preparations were typically short burst responses (Fig. 2 and Fig. 7A, bottom trace). In contrast, intact preparations showed CRs that were typically sustained throughout the duration of the CS. Preparations in which the cerebellum/red nucleus had been removed, but that had an intact brain stem, showed some CRs that were bursts and a majority that were sustained (Fig. 7A, top trace). A summary of the average timing of the CRs in the three different preparations tested is shown schematically in Fig. 7B. The short burst responses produced by the brain stem tissue also explains why the amplitude of the CRs were significantly reduced compared with the other preparations (Fig. 6B). These data suggest that the integrity of the sustained component of the UR is not required to obtain abducens nerve CRs. Furthermore, the caudal brain stem, at least in this reduced preparation, is not a site of acquisition, but it contributes to maintaining the activity of both the CR and UR. Without the circuitry in the caudal brain stem, the CRs are not sustained and subsequently appear as short, maladaptive, bursts. These results do not imply that this region of the brain stem is not involved in acquisition in more intact preparations, rather the possibility remains that the learned and reflex responses share pathways in the caudal brain stem. Finally, the shift in the onset latency of CRs to later periods in the CS with training (Fig. 5B) is not different between preparations with cerebellar lesions and those with additional removal of brain stem tissue. Thus removal of the caudal brain stem does not further exacerbate the
effects on CR onset latency observed after removal of the cerebellum. Taken together, these findings suggest that classical conditioning may occur locally in the abducens nerve reflex circuitry. Whether this occurs in intact preparations is uncertain. Moreover, learning takes place elsewhere in the circuit as the learned timing of CRs appears to be controlled by the cerebellum and red nucleus.

Classical conditioning in a reduced brain stem preparation

What are the neural substrates for acquisition of abducens nerve CRs in this thick section of brain stem tissue? Recent neuroanatomical findings in the turtle have described the projection patterns of both the trigeminal and the posterior eighth nerves (Herrick and Keifer 1998) and may provide some insight into this question. In addition to the cerebellar cortex, which is not present in these preparations, there are two sites of direct CS-US convergence in the abducens nerve reflex circuitry itself. Converging inputs occur on neurons of the principal sensory trigeminal nucleus and on both the principal and accessory abducens motoneuclae. Thus the anatomic substrates for associative learning to occur in this circuitry are in place. It is also particularly relevant with regard to the present findings that pharmacological studies have shown that these circuits use glutamate neurotransmission acting through both N-methyl-D-aspartate (NMDA) and non-NMDA receptor subtypes (Keifer 1993). These receptors are implicated in mechanisms underlying Hebb-like–synapse modification in other models of learning (Malenka 1994; Murphy and Glanzman 1997). These data allow the hypothesis that the mechanisms underlying classical conditioning in this reduced preparation are occurring at principal sensory trigeminal interneurons and/or at the abducens motoneurons directly. The pathways involving the principal sensory trigeminal nucleus, however, are as yet unspecified and potential connections with the abducens motoneurons will require definition. With the use of this reduced in vitro brain stem preparation from the turtle, it appears that mechanisms of CR acquisition can be studied somewhat independently of the more complex timing mechanisms that arise from the cerebellorubral system.

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