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Neuroscience Research 56 (2006) 363–371
Neuroscience Research
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Reticulo-collicular and spino-collicular projections involved in eye and eyelid movements during the blink reflex

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Received 3 April 2006; accepted 14 August 2006
Available online 14 September 2006

Abstract

Reflex blinking provides a useful experimental tool for various functional studies on the peripheral and central nervous system, yet the neuronal circuitry underlying this reflex is not precisely known. In the present study, we investigated as to whether neurons in the reticular formation and rostral cervical spinal cord (C1) may be involved in the blink reflex in rats. To this end we investigated c-Fos expression in these areas following supraorbital nerve stimulation combined with retrograde tracing of gold conjugated horse radish peroxidase (Gold–HRP) from the superior colliculus. We observed many double labeled neurons in the parvocellular reticular nucleus, medullary reticular formation, and laminae IV and V of C1. Thus, these brain regions contain neurons that may be involved in blink reflexes as well as eye movements, because they both can be activated following peri-orbital stimulation and project to the superior colliculus. Consequently, we suggest that the medullary reticular formation and C1 region play a central role in the coordination of eye and eyelid movements during reflex blinking.

Keywords: Superior colliculus; Reticular formation; Spinal cord; Blink reflex; Eye and eyelid movements; Blink generator

1. Introduction

Blinking is a process in which eyes and eyelids act in concert. After onset of eyelid closure the eyes move characteristically nasal downward and then lateral upward (Evinger et al., 1984; Collewijn et al., 1985). The pre-motor neuronal circuits controlling the simultaneous eye and eyelid movements during the blink reflex have been investigated intensively (Holstege et al., 1986a; Van Ham and Yeo, 1996a,b; Morcuende et al., 2002; Zerari-Mailly et al., 2003; Cruccu et al., 2005). Trigeminal blinks can be elicited by supraorbital (SO) nerve or corneal stimulation (Evinger et al., 1984; Gruart et al., 1995; VanderWerf et al., 2003). In humans, EMG recordings of the eyelid-closing orbicularis oculi muscle shows two responses after electrical stimulation of the SO nerve (Kugelberg, 1952; Aramideh et al., 2002). The brief early response R1 is unilateral and the large late response R2 bilateral and corresponds with the actual eyelid movement in humans. Guinea pigs, cats and rats however show bilateral R1 and R2 responses upon moderate-intensity electrical stimulation. In guinea pigs, both the R1 and R2 contribute significantly to eyelid movement during the blink (Pellegrini et al., 1995). The circuitry regulating trigeminal blinks is short, nonetheless not uncomplicated. The orbicularis oculi motoneurons in the facial motor nucleus are innervated through three different pathways. The shortest (Fig. 1, pathway 1), direct circuit involves the sensory trigeminal complex (STC) which directly projects to the facial motor nucleus (Jacquin et al., 1993; Van Ham and Yeo, 1996b). From the STC the other two indirect pathways arise. The first by way of the reticular formation (Fig. 1, pathway 2), the second (Fig. 1, pathway 3) via the rostral

Abbreviations: C1, rostral part of the cervical spinal cord; Gold–HRP, colloidal gold apo–horseradishperoxidase complex; MdD, dorsal medullary reticular nucleus; PCRt, parvocellular reticular nucleus; SC, superior colliculus; SO nerve, supraorbital nerve; STC, sensory trigeminal complex

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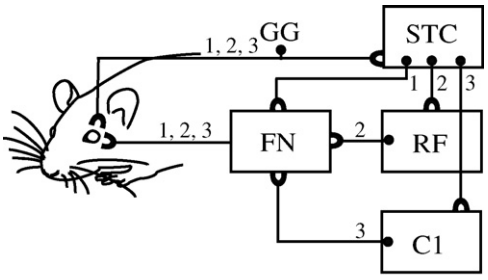
0168-0102/$ – see front matter
doi:10.1016/j.neures.2006.08.005



1. Short direct pathway via the STC and FN.
2. Indirect pathway via the STC, RF and FN.
3. Indirect pathway via the STC, C1 and FN.

Fig. 1. The blink reflex circuit. Three pathways can be distinguished (1–3). Abbreviations: gasserian ganglion, GG; sensory trigeminal complex, STC; facial motor nucleus, FN; reticular formation, RF; rostral part of the cervical spinal cord, C1.

cervical spinal cord (C1) (Zerari-Mailly et al., 2003). The indirect pathways were studied in the cat (Holstege et al., 1986b), guinea pig (Pellegrini et al., 1995), rabbit (Van Ham and Yeo, 1996a,b) and rat (Zerari-Mailly et al., 2003). Higher brain regions, like the basal ganglia, can also influence reflex blinking through different brainstem structures (Basso et al., 1996).

Excitability of the blink can be modulated by descending cortical projections via the thalamus and superior colliculus (SC) via tecto-reticular projections (Basso et al., 1996). The SC is important for eye and head movements as well as for many other sensory motor functions including the blink reflex (Goossens and Van Opstal, 2000a,b; Ndiaye et al., 2002; King, 2004). SC involvement in the blink reflex was also shown with experiments in the monkey (Gnadt et al., 1997) and rat (Basso et al., 1996) where electrical micro-stimulation of the SC suppressed the trigeminal blink reflex. Hemi-facial paralysis patients have impaired eye and eyelid movements during blinking, while the eye moves normal during saccades and smooth pursuit (VanderWerf et al. unpublished results). This implies that the SC as eye movement generator is not affected; therefore, a separate structure projecting to the SC must initiate eye movement during the blink. Recently, the latero-caudal SC was shown to receive input from two reticular areas; the ventral part of the parvocellular reticular nucleus (PCRt) and the dorsal part of the medullary reticular nucleus (MdD) (Smit et al., 2005). Subsequently, the question arose whether the coordination of eye movements during blinking is regulated by the reticular formation and/or C1 and thus how the projections towards the SC are arranged.

Since timing of the stereotypical eye and eyelid movement during the blink is known to be very precise (Bour et al., 2000) a common neuronal structure must initiate the coordination of both movements. We hypothesize that different areas in the RF can generate eye and eyelid movement associated with the blink reflex. A reticular area that receives SO nerve input and contains neurons that project to the facial motor nucleus as well as neurons that project to the SC could be the common neuronal structure regulating this phenomenon.

The aim of the present study was to clarify the role of the reticular formation, spinal cord and SC in eye movement during reflex blinks and define the location of a central neural origin of the eye and eyelid components of the blink reflex. To this end we examined the functional and topographical relationships between the spino- and or reticulo-collicular connections and the primary afferent inputs from the eyelids (i.e., the SO nerve). Localization of cells in functional pathways in the nervous system was achieved through immunohistochemical detection of cellular counterpart of the immediate early gene c-Fos. The c-Fos gene encodes the nuclear protein c-Fos that is rapidly and transiently expressed in neurons in response to various peripheral stimuli (Hunt et al., 1987; Sheng and Greenberg, 1990). The development and use of this method has been reviewed extensively (Armstrong and Montminy, 1993; Hoffman and Lyo, 2002; Munglani and Hunt, 1995; Sheng and Greenberg, 1990). Since c-Fos activation is a well-established high resolution metabolic marker for polysynaptic pathway tracing in the brain (Dragunow and Faull, 1989), in the current study expression of the c-Fos protein was evaluated after electrical SO nerve stimulation normally eliciting trigeminal blinks. In addition to this experimental series, a retrograde tracer, the colloidal gold apo-horseradish peroxidase complex (Gold–HRP), was also employed (Ménétrey, 1985). The combination of c-Fos expression following electrical SO nerve stimulation and Gold–HRP micro-injections in the SC were used to identify reticular and spinal neurons involved in the trigeminal blink reflex and neurons that project to the SC, respectively. These experiments may identify neurons in the pontine and medullary reticular formation involved in the organisation of the blink reflex.

2. Materials and methods

2.1. Animals

For the experiments, nine adult Sprague–Dawley rats were used. Animals were anesthetized with an intraperitoneal sodium pentobarbital injection (50 mg/kg), a very suitable anesthetics for c-Fos expression studies in rats (Takayama et al., 1994). During surgery, animals were placed in a stereotaxic apparatus. Gold–HRP injections were made in the right side of the brain, electrical SO nerve stimulation on the left. The micro-injection were aimed at the latero-caudal SC, since it has been shown that this portion of the SC has reciprocal connections with the STC and is involved in the blink reflex (Ndiaye et al., 2002; Dauvergne et al., 2004). To determine the effects of anaesthetics and surgical procedures on c-Fos expression, three control animals were anesthetized and sham operated similar to the study of Zerari-Mailly et al. (2003). Six animals received both a Gold–HRP injection and electrical stimulation. All experiments were conducted following the "principles of laboratory animal care" (NIH publication No. 86-23, revised 1985) and French law on the protection of animals.

2.2. Experimental procedures

2.2.1. Colloidal gold apo–horseradishperoxidase complex (Gold–HRP) injection

Gold–HRP solution (Ménétrey, 1985) was pressure injected into the SC according to stereotaxic coordinates defined by Paxinos and Watson (1986). Six micro-injections (0.1–0.15 μl) were made into the right SC 3 days prior to electrical stimulation of the SO nerve. The exact locations of the injections are given in Fig. 2.

2.2.2. Electrical stimulation

In six rats, the main branches of the left SO nerve were dissected, cut distally, placed over a pair of silver hook electrodes and covered with mineral

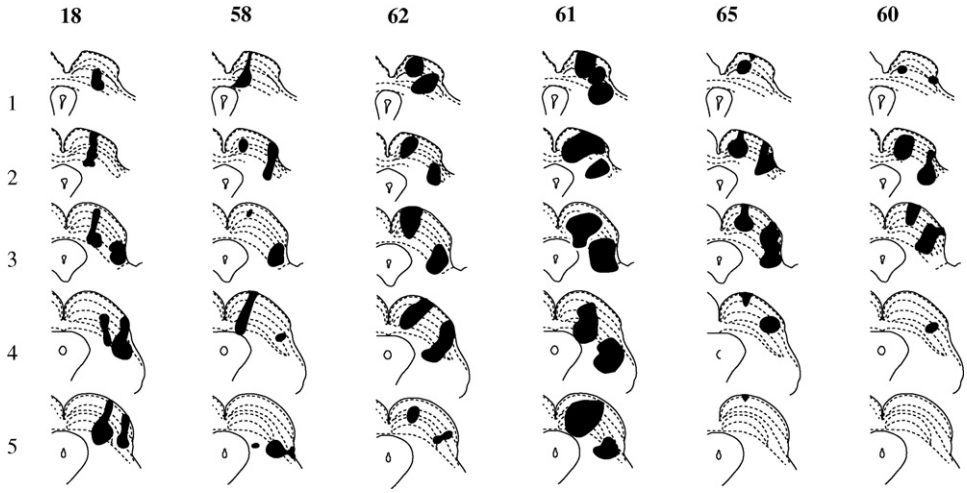


Fig. 2. Schematic drawing of transverse sections of the superior colliculus (from rostral 1 to caudal 5) depicting the Gold–HRP injection sites in the six rats.

oil. The nerve was stimulated with three pulse bursts (300 Hz, 0.1 ms duration; repeated every 900 ms for 1 h). The intensity of the stimulation was 1.5 times the blink reflex threshold value (0.1–1.0 mA; up to 1 mA in case 18). Presumably nociceptive A and C fibers were not stimulated at this intensity as Ellrich et al. (2001) showed that the nociceptive R3 component of the blink was not elicited after stimulation under 2.9 times the detection threshold of a blink. Furthermore, no neck movement was observed at the chosen stimulus. One hour after stimulation the animals were perfused with 500 ml phosphate-buffered saline (PBS, pH 7.4) and 500 ml cooled fixative (4% paraformaldehyde in PBS). The brains were stored in a 30% sucrose PBS solution at 4 °C for 48 h. Forty micrometer thick slices were cut with a freezing microtome.

2.3. Histochemical procedures

2.3.1. Gold–HRP histochemistry

Sections were processed with a silver intensification method to reveal the protein gold complex. The procedures have been described by Zerari-Mailly et al. (2003).

2.3.2. c-Fos immunohistochemistry

Detailed procedures for visualization of c-Fos expression were previously described (Zerari-Mailly et al., 2003).

2.4. Data analysis

2.4.1. Illustrations

Drawings were made with a camera lucida and imported into Adobe Illustrator 10.0. Photomicrographs were taken with a Leitz Diaplan photomicroscope and processed with Adobe Photoshop 6.0 (Adobe systems Inc., San Jose, CA).

2.4.2. Cell counting

The number of single labeled c-Fos and Gold–HRP labeled neurons as well as double labeled neurons was counted in three control and six stimulated rats. Labeled neurons were counted in the ponto-medullary reticular formation. Slices were included from the rostral facial motor nucleus until just above the spino-medullary junction. Seven sections were selected just below the spino-medullary junction, for counting c-Fos neurons in the C1.

3. Results

3.1. c-Fos labeling

SO nerve stimulation induced a large increase in the number of c-Fos positive neurons compared to control animals and numerous c-Fos positive neurons were observed throughout the pontomedullary reticular formation (Table 1; Fig. 3) and C1 segment of the spinal cord (Table 1; Fig. 4). The locations of labeled areas were similar on the ipsi-and contralateral sides, however the number of c-Fos positive neurons was consistently higher at the stimulated side. Large numbers of c-Fos positive neurons were found in three reticular areas: the PCRt, the MdD, and the C1; 700, 475 and 934, respectively (Table 1). Whereas, in control animals these numbers were 64, 185 and 676, respectively (Table 1). The highest increase of c-Fos positive neurons was found in the PCRt (1000%, Table 1).

In the pontine reticular formation (Fig. 3, cases 18A, 62A, 65A, level 1–3) c-Fos positive neurons were concentrated ventral and medial to the STC. Smaller groups of c-Fos positive neurons were located around the trigeminal motor nucleus and medial to the VIIth cranial nerve. In addition, c-Fos labeled neurons were observed near the oral subnucleus of the STC. In the medullary reticular formation (Fig. 3, cases 18A, 62A, 65A,

Table 1
Number of c-Fos positive neurons found in the parvocellular reticular nucleus (PCRt), dorsal medullary reticular formation (MdD) and rostral part of the cervical spinal cord (C1) in control and experimental animals

	PCRt	MdD	C1
Control			
22	34	197	659
24	43	162	644
36	114	195	725
Average	64	185	676
Experiment			
18	850	418	1200
58	450	302	739
60	457	363	879
61	819	633	918
62	913	632	–
65	713	502	–
Average	700	475	934
% Increase vs. control	1000	157	38

Countings were made ipsilateral to the stimulated side.

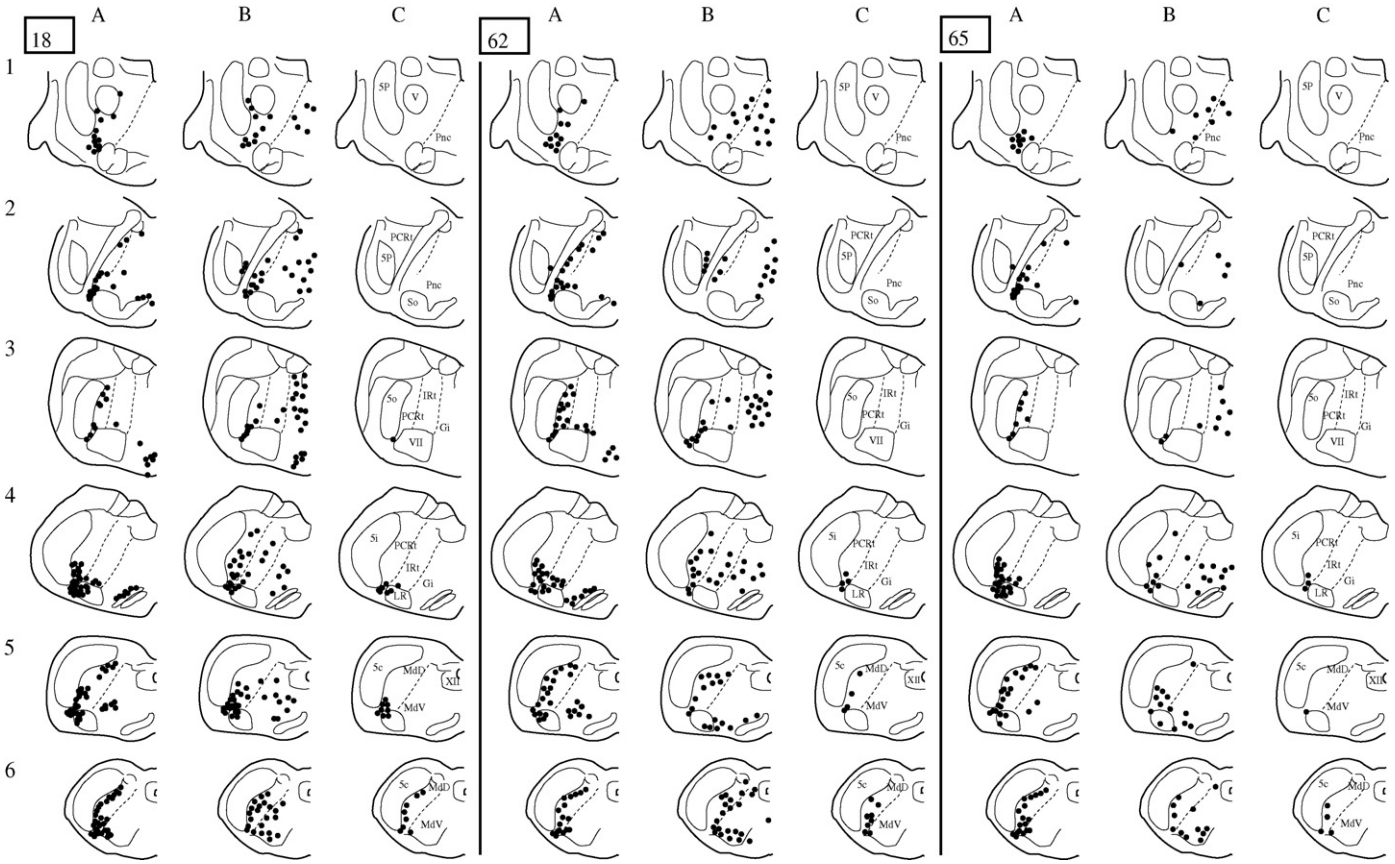


Fig. 3. Schematic drawing of the pontomedullary reticular formation from rostral level 1 to caudal level 6. Black dots indicate the location in which labeled neurons were found in cases 18, 62 and 65; total numbers of labeled neurons are shown in Table 1. (A) The distribution of c-Fos positive neurons after supraorbital (SO) nerve stimulation. (B) The distribution of Gold–HRP labeled neurons following micro-injections in the caudo-lateral portion of the superior colliculus (SC). (C) The distribution of c-Fos positive/Gold–HRP double labeled neurons following SO nerve stimulation and micro-injections in the caudo-lateral SC portion. Abbreviations: principal nucleus of the sensory trigeminal complex, 5P; oral subnucleus of the sensory trigeminal complex, 5o; interpolar subnucleus of the sensory trigeminal complex, 5i; caudal subnucleus of the sensory trigeminal complex, 5c; gigantocellular reticular nucleus, Gi; intermediate reticular nucleus, IRt; lateral reticular nucleus, LR; dorsal medullary reticular nucleus, MdD; ventral medullary reticular formation, MdV; parvocellular reticular nucleus, PCRt; caudal pontine reticular nucleus, Pnc; superior olivary nucleus, So; trigeminal motor nucleus, V; facial motor nucleus, VII; hypoglossal motor nucleus, XII.

level 4–6), most c-Fos positive neurons were found ventral in the caudal PCRt, between the interpolaris subnucleus of the STC and lateral reticular nucleus, and in the lateral portion of the MdD. c-Fos positive neurons were also seen laterally within the lateral reticular nucleus. In addition, some c-Fos positive neurons were observed in the intermediate reticular field just above the facial motor nucleus and/or the lateral reticular nucleus in the gigantocellular reticular nucleus, adjacent to the inferior olive, and in the ventral medullary reticular nucleus (Fig. 3, cases 18A, 62A, 65A).

In the C1 (Fig. 4, cases 18A, 58A, 61A) c-Fos positive neurons were found in lamina I–VI with a concentration of labeled neurons ventro-lateral in the superficial laminae. A comparison with control animals revealed that most c-Fos positive neurons were located within laminae III–V.

4. Gold–HRP labeling

In six experiments, Gold–HRP injections were made in the SC contralateral to the side of SO nerve stimulation (Fig. 2). In four injections, the rostrocaudal extent of the SC was impregnated by the tracer: the injection sites comprised the central and caudo-lateral (case 18), medial and caudo-lateral (case 58) and entire SC (cases 62 and 61). In two experiments, the injection sites were restricted to the rostral SC (cases 65 and 60). When the injection site encompassed the caudo-lateral portion of the SC numerous Gold–HRP labeled neurons were located throughout the pontomedullary reticular formation (Table 2; Fig. 3, cases 18B, 62B, 65B) and C1 (Table 2; Fig. 4, cases 18B, 58B, 61B). Labeled neurons were predominantly found contralateral to the side of Gold–HRP injection. The number of labeled neurons increased with a larger injection site. The PCRt as well as the MdD contained approximately three times more labeled neurons than C1 (Table 2).

In the pontine reticular formation (Fig. 3, cases 18B, 62B, 65B, level 1–3) numerous labeled neurons were observed around the trigeminal motor nucleus, around the VIIth nerve, and wedged between the oral subnucleus of the STC and the facial motor nucleus. Cases 60 and 65 demonstrate that if the injection site does not comprise the most caudal lateral SC portion less neurons are labeled, which is most obvious in the

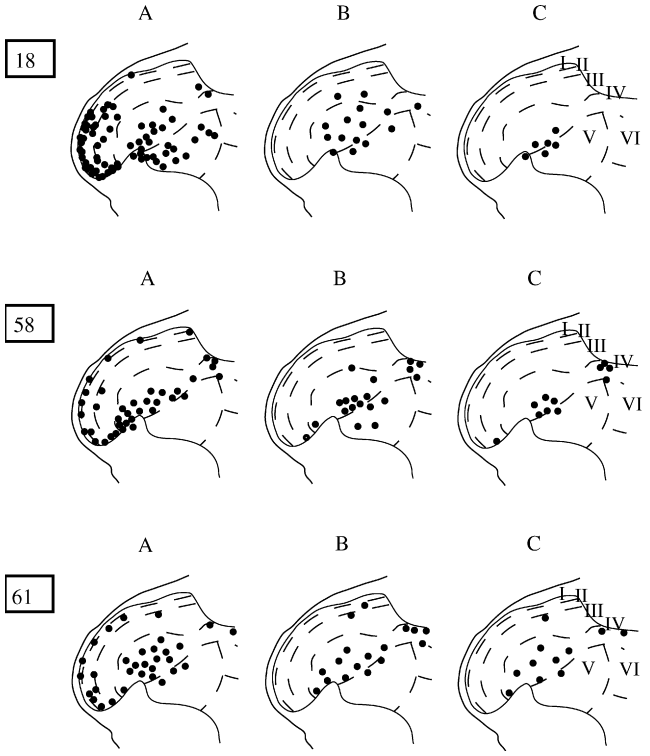


Fig. 4. Schematic drawing of the rostral part of the cervical spinal cord showing the distribution of labeled neurons in cases 18, 58 and 61. Black dots represent the location of labeled c-Fos positive or Gold–HRP neurons from the dorsal layer I to the ventral layer VI; total numbers of neurons are shown in Table 1. (A) The distribution of c-Fos positive neurons after supraorbital (SO) nerve stimulation. (B) The distribution of Gold–HRP labeled neurons following micro-injections in the caudo-lateral portion of the superior colliculus (SC). (C) The distribution of c-Fos positive/Gold–HRP double labeled neurons following SO nerve stimulation and micro-injections in the caudo-lateral SC portion.

small amount of labeled neurons in the PCRt (Fig. 3, case 65B, level 1–4).

In the medullar reticular formation (Fig. 3, cases 18B, 62B, 65B, level 4–6), labeled neurons were predominantly found in the PCRt and the MdD. Neurons were also labeled in the medial and intermediate reticular formation. In case 60, which was injected in the lateral portion of the rostral SC, numerous labeled neurons occupied the medial reticular formation. Few labeled neurons were found in the lateral reticular formation, i.e. in areas involved in the blink reflex.

In the C1 (Fig. 4, cases 18B, 58B, 61B) Gold–HRP labeled neurons were observed in laminae III–VI, with predominance in laminae III and IV. Gold–HRP labeled neurons were virtually absent in laminae I and II.

Table 2
Number of Gold–HRP and double labeled neurons in the parvocellular reticular nucleus (PCRt), dorsal medullary reticular nucleus (MdD) and rostral part of the cervical spinal cord (C1) counted ipsilateral to the stimulated side

Experiment	Gold–HRP			Double labeled		
	PCRt	MdD	C1	PCRt	MdD	C1
18	215	195	51	76	85	7
58	160	99	46	35	55	11
60	6	0	1	0	0	0
61	177	134	44	62	84	10
62	135	168	–	28	62	–
65	105	150	–	25	15	–
Average	133	124	36	38	50	7
% Gold–HRP				28	40	20
% c-Fos				5	11	0,7

Percentage of Gold–HRP = percentage of Gold–HRP labeled neurons that was double labelled; percentage of c–Fos = percentage of c-Fos positive neurons that was double labeled.

5. Double labeling

c-Fos/Gold–HRP double labeled neurons were observed primarily in the ventral PCRt, MdD (Fig. 3, cases 18C, 62C, 65C) and C1 (Fig. 4, cases 18C, 58C, 61C). In case 60, with a rostral injection (Fig. 2), no double labeled neurons were present. The distribution of double labeled neurons in the reticular formation was bilateral. The largest number of double labeled neurons was found contralateral to the side of injection and ipsilateral to the side of SO nerve stimulation. The MdD not only contained the highest number of double labeled neurons but the relative proportion of the two neuron populations that are either activated by SO nerve stimulation or project to the SC was also the highest (i.e. twice the amount of C1 and 1.5 that of PCRt; Table 2).

In the pontine reticular formation (Fig. 3, cases 18C, 62C, 65C, level 1–3) practically no double labeled neurons were observed. Some double labeled neurons were observed between the oral subnucleus of STC and facial motor nucleus (Fig. 3, case 18C, level 3).

In the medullary reticular formation (Fig. 3, cases 18C, 62C, 65C, level 4–6), double labeled neurons are observed in the PCRt (Fig. 5a) and MdD (Fig. 5b). In the PCRt, double labeled neurons are found in the ventral part, mostly between the interpolar subnucleus of the STC and the lateral reticular nucleus. In the MdD, double labeled neurons were found adjacent to the lateral reticular nucleus and medial to the caudal subnucleus of the STC. In case 65, relatively few double labeled neurons were found in the MdD.

In the C1, double labeled neurons were mainly found in laminae IV and V (Fig. 5c), additionally a few double labeled neurons were found in lamina III (Fig. 4, cases 18C, 58C, 61C).

6. Discussion

In the current study we aimed to reveal reticulo-collicular (Fig. 6; pathway 1) and spino-collicular (Fig. 6; pathway 2) projections involved in reflex blinking. In these experiments, c-Fos immunohistochemistry following SO nerve stimulation (Fig. 6 thick grey dashed lines) and Gold–HRP injections into the SC (Fig. 6 thick black dashed lines) were combined. We determined specific areas in the reticular formation and C1.

The distribution patterns of c-Fos neurons following SO nerve stimulation were determined in the pontomedullary reticular formation and the C1. c-Fos neurons were predominantly observed in the lateral reticular formation and laminae

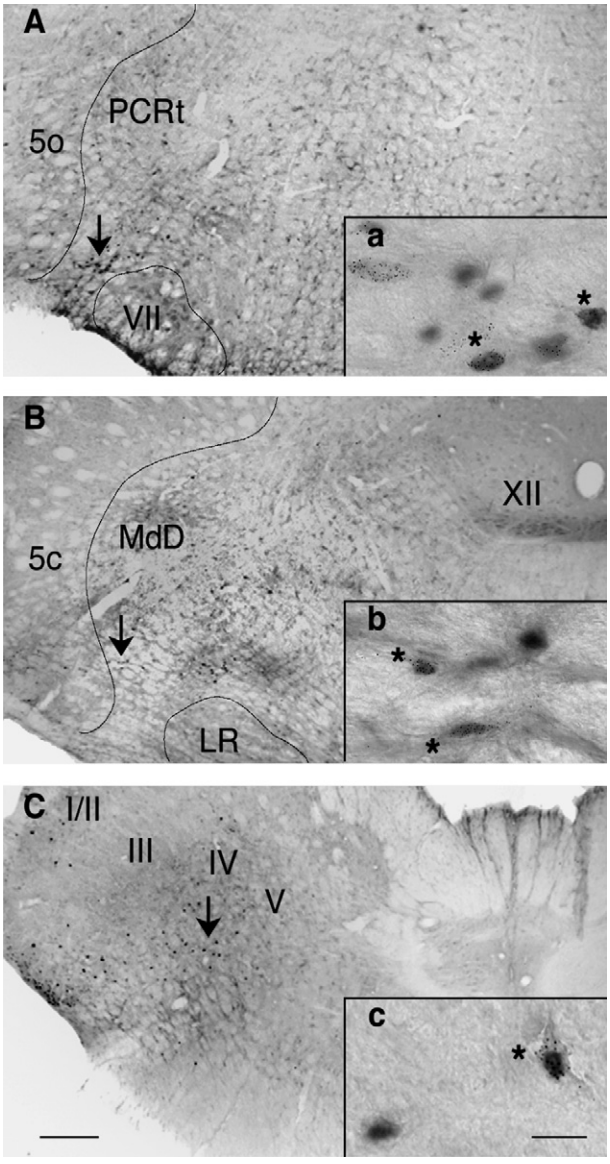
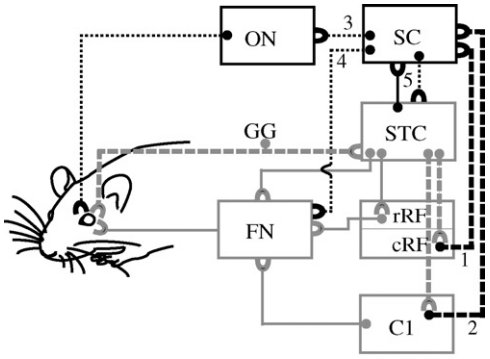


Fig. 5. Photomicrographs of transverse slices from case 18 containing double labeled neurons in (A) the parvocellular reticular nucleus, (B) dorsal medullary reticular nucleus and (C) rostral part of the cervical spinal cord. a–c are magnifications of areas in A–C indicated by arrows. Asterisks are placed near double labeled neurons in the magnifications. Abbreviations: oral subnucleus of the sensory trigeminal complex, 5o; caudal subnucleus of the sensory trigeminal complex, 5c; lateral reticular nucleus, LR; parvocellular reticular nucleus, PCRt; dorsal medullary reticular nucleus, MdD; facial motor nucleus, VII; hypoglossal motor nucleus, XII; laminae of the C1 cervical spinal cord, I–V. Scale bar = 200 μm for A–C/10 μm for a–c.

IV and V of C1. Possible c-Fos inducers other than electrical stimulation of SO nerve were not found: anaesthesia, surgical manipulation and placement of electrodes upon the SO nerve or tracer injections into the SC did not induce c-Fos expression in the examined reticular formation areas. In addition, c-Fos expression after SO nerve stimulation was not induced by a non-specific effect like nociceptive stimulation of C fibers. If unmyelinated C fibers were stimulated most c-Fos would have been expressed in lamina II of the C1, where very little c-Fos expression was found in this study (Pellegrini et al., 1995).



1. Reticulo-collicular projection.
2. Spino-collicular projection.
3. Colliculo-ocolomotor projection.
4. Colliculo-facial projection.
5. Colliculo-trigeminal projection.
   Trigemino-collicular projection.

Fig. 6. The blink reflex circuit (grey) expanded with structures/projections involved in eye movement during the blink reflex (black). The projections examined in the current study are indicated by thick grey (supraorbital nerve stimulation) and black (Gold–HRP injection) dashed lines. Double labeled neurons are located in caudal reticular formation (cRF) and cervical spinal cord (C1). The projections in the scheme are based on findings of the current and other studies of Smit et al. (2005), the reticulo-collicular and spino-collicular projections (1 and 2), of Goossens and Van Opstal (2000b), the colliculo-oculomotor projections (3), of Dauvergne et al. (2004), the colliculo-trigeminal and colliculo-facial projections (4 and 5), of Ndiaye et al. (2002), the colliculo-trigeminal and trigemino-collicular projections (5), and of Zerari-Mailly (2003), the trigemino-facial, trigemino-reticular and reticulo-facial projections (grey lines). Abbreviations: gasserian ganglion, GG; sensory trigeminal complex, STC; facial motor nucleus, FN; rostral reticular formation, rRF; caudal reticular formation, cRF; rostral part of the cervical spinal cord, C1; superior colliculus, SC; oculomotor nuclei, ON.

In the present experiments, the SO nerve stimulation induced c-Fos expression in trigeminal neurons, confirming labeled areas described by Ndiaye et al. (2002) and Zerari-Mailly et al. (2003). However, despite known disynaptical facial motoneuron activation during experimental conditions, no c-Fos labeling was observed in the facial motor nucleus. Absence of labeling in regions that are activated has been shown in several studies such as motoneurons or other neurons in the dorsal root ganglia or the substantia nigra (Hunt et al., 1987; Dragunow and Faull, 1989; Carr et al., 1995; Dai et al., 2005). Moreover, Dragunow and Faull (1989) suggested that (moto)neurons might lack the required biochemical messengers regulating c-Fos activation. Subsequently, facial motoneurons that are active under specific conditions can fail to express c-Fos.

The present data demonstrated that most of the labeled c-Fos neurons appeared rostrally around the motor trigeminal nucleus, wedged between the facial nerve and the superior olivary nucleus, and in the PCRt. Caudally, labeled c-Fos neurons were observed in the ventral PCRt and scattered within the MdD. In the C1, c-Fos neurons were found in laminae I–VI concentrated in lamina IV and V. In rats, some of these structures are pre-motor areas for reflex blinking.

In a previous neuroanatomical study on the eyelid movement during reflex blinking the SO nerve was stimulated and the

facial motor nucleus injected with Gold–HRP (Zerari-Mailly et al., 2003). Double labeled neurons, which receive SO nerve input and both project to the facial motor nucleus, were found in the ponto-medullary reticular formation and dorsal horn of the C1. A summary diagram of the distribution pattern of these double labeled neurons is given in Fig. 7 (grey areas). When the injection sites comprised the ipsilateral dorsal facial motor nucleus, containing orbicularis oculi motoneurons, the double labeled neurons were found in three different reticular areas which might hence be part of the indirect pathway of the trigeminal blink reflex (Fig. 1, pathways 2 and 3, Fig. 6 thick grey dashed lines). The first area was in the pontine reticular formation, rostral around fiber bundles of the VIIth cranial nerve and the trigeminal motor nucleus (Fig. 7, grey area, level 1–2). This pontine area near the trigeminal motor nucleus is similar to the pre-motor area found by Holstege et al. (1986b). In agreement with a study by Mogoseanu et al. (1994) monosynaptic projections were shown from the PCRt to facial motoneurons, confirming the idea of a specific eyelid control area in the pontine reticular formation of rats. The second reticular area was the caudal MdD in the medullary reticular formation. (Fig. 7, grey area, levels 5 and 6). The third area was in C1 spinal cord, comprising the inner lamina IV and outer lamina V (Fig. 7, grey area, level 7).

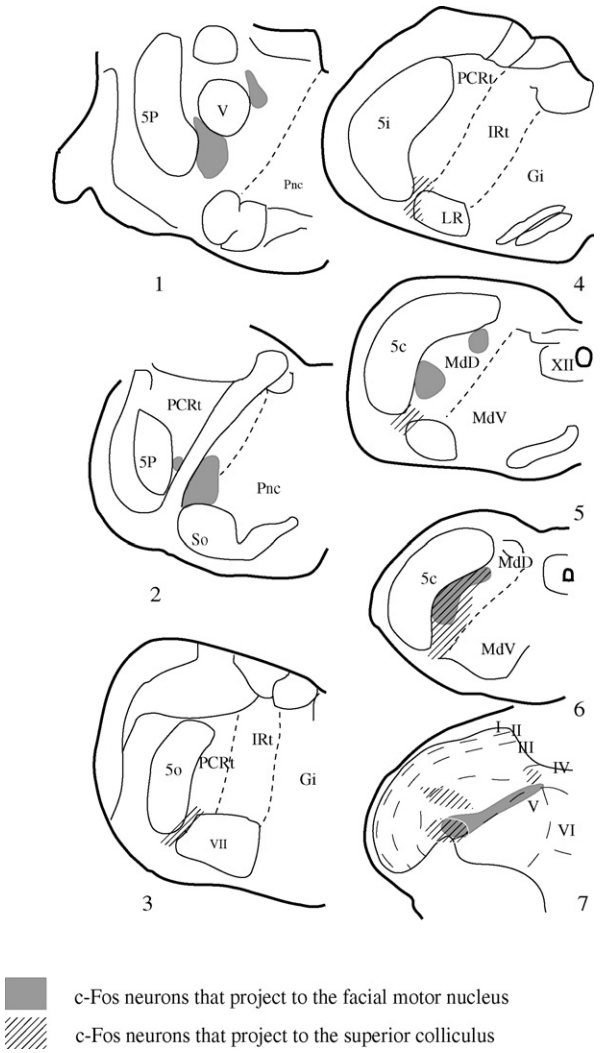


Fig. 7. Summary diagram showing the pontomedullary reticular formation and rostral part of the cervical spinal cord (C1) from rostral level 1 to caudal level 7. The location of neurons that receive supraorbital nerve input and project to the facial motor nucleus are indicated by grey areas, the location of neurons projecting to the superior colliculus are indicated by hatched areas. Partial overlap of two areas is indicated; one in the dorsal medullary reticular nucleus and the other in laminae IV and V of the C1. Abbreviations: principal nucleus of the sensory trigeminal complex, 5P; oral subnucleus of the sensory trigeminal complex, 5o; interpolar subnucleus of the sensory trigeminal complex, 5i; caudal subnucleus of the sensory trigeminal complex, 5c; gigantocellular reticular nucleus, Gi; intermediate reticular nucleus, IRt; lateral reticular nucleus, LR; dorsal medullary reticular nucleus, MdD; ventral medullary reticular formation, MdV; parvocellular reticular nucleus, PCRt; caudal pontine reticular nucleus, Pnc; superior olivary nucleus, So; trigeminal motor nucleus, V; facial motor nucleus, VII; hypoglossal motor nucleus, XII.

In the present study, c-Fos immunohistochemistry following SO nerve stimulation and Gold–HRP injections in the SC were combined. The distribution pattern of c-Fos neurons which receive SO nerve input and project to the SC is given in Fig. 7 (hatched areas). Double labeled neurons were found in the medullary reticular formation, but rarely in the pontine reticular formation. In the medullary reticular formation two areas contained a population of double labeled neurons, the ventral PCRt (Fig. 7, hatched area, levels 3 and 4), the ventral MdD (Fig. 7, hatched area, level 5), and more caudal the ventral as well as the dorso-lateral MdD (Fig. 7, hatched area, level 6). Double labeled neurons were also found in laminae III–V of the C1 (Fig. 7, hatched area, level 7).

Comparison of the distribution of c-Fos reticulo-facial and reticulo-collicular neurons shows that two neuronal populations can be distinguished within the PCRt. A rostral area, which projects to the facial motor nucleus (Fig. 6, level 1–2), confirms that the PCRt is involved in eyelid movements. An area in the ventrocaudal PCRt projects to the SC (Fig. 7, level 3–4), indicating that the PCRt is also involved in eye movements during blinking. Thus, pre-motor areas of the eye and eyelid movement during reflex blinking are separated in the PCRt. The lack of a substantial number of neurons in the PCRt that both project to the SC and the facial motor nucleus and are innervated by the SO-nerve in the current study makes it unlikely that this area is a candidate for a common eye and eyelid movement generator.

Most c-Fos labeled neurons that project to the SC were found in the MdD, implying an important role for this area in the eye movement during the blink. In the rostral MdD, neurons projecting to the SC are located ventrally and those projecting to the facial motor nucleus are located dorsally (Fig. 7, level 5). In the caudal MdD the neuron populations of the two studies overlap (Fig. 7, level 6). This overlapping area might be involved in motor control of the orbicularis oculi muscle as well as in eye movement control through the SC projection. Another possibility is that this area might be involved in the control system during saccadic movement. It has been suggested that blinking is linked to saccadic gaze shifts by a common premotor drive (Evinger et al., 1994) which might be comprised by this area.

The ventral MdD is a functionally complex and heterogeneous brain area which is involved in autonomic functions,

motor reactions and pain responses (Cobos et al., 2003). This area encompasses main autonomic functions like regulation of cardio-vascular and respiratory functions. The caudal ventro-lateral medulla also projects to orofacial motor nuclei and participates in motor control through cerebellar and rubral connections (Jones, 1995). A recent MRI study by Cruccu et al. (2005) on patients with brainstem lesions gave new insights into the roles of different structures during the early (R1) and late (R2) components of blinking. Most lesions affecting the R2 response were found in the dorso-lateral medulla at the level of the inferior olive. This area corresponds precisely with the area containing most double labeled neurons in the MdD in the current study.

A second overlapping area which contains neurons that are innervated by the SO nerve and project to the SC or facial motor nucleus is located in laminae IV and V of the C1 (Fig. 7, level 7). Like the MdD, this area might be involved in the coordination of eye and eyelid movement during blinking or even saccadic gaze shifts. The trigeminal system was proposed by Goossens and Van Opstal (2000a,b) as a candidate structure for saccade inhibition during reflex blinking, because the short latency with which a saccadic perturbation occurs indicates a short circuit. This pleads for a role of the non-overlapping hatched area in the C1 (Fig. 7, level 7) in saccadic control, as C1 is the caudal prolongation of the STC.

The C1 receives direct trigeminal eye blink input (Van Ham and Yeo, 1996b) and is an important area for blink initiation and modulation, which can be demonstrated by suppression of corneal-evoked blinks through micro-stimulation of the C1 area. (Cruccu et al., 2005; Henriquez and Evinger, 2005). The C1, like the MdD, is also a relay centre in the R2 response; Pellegrini et al. (1995) observed in guinea pigs that the R2 blink response, but not the R1 response, was eliminated after hemi-section at the level of the C1. As blink related C1 projections towards the SC were found in the current study, the C1 might, serve as an initiator of the eye movement accompanying the eyelid movement of the R2 component.

The distribution of reticulo-collicular and reticulo-facial neurons receiving input from SO nerve overlap in the MdD and the C1. This mixed group of reticulo-collicular and reticulo-facial neurons is a good candidate to constitute a ‘‘blink generator’’ regulating the precise timing of eye and eyelid movement. An anterograde tracing study in the rat which revealed projections from the red nucleus, *the* output nucleus of the cerebellum, towards the MdD, supports this hypothesis (Cobos et al., 2003). The generator might be composed of different premotor structures located in the brainstem; another possibility is one area stretching from the MdD to the C1. This area would correspond with the area described by Kimura and Lyon (1972) responsible for the R2 response, conducted through the descending spinal tract, in the lower part of the medulla oblongata. Future studies will have to reveal whether the overlapping areas described here in the caudal MdD and C1 are indeed common areas from where projections originate to facilitate both the eye and eyelid component of the blink reflex.

Acknowledgement

This work was supported by NWO grant VGP 95-398, EU-SENSOPAC.

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