Effects of dopamine D$_1$ and D$_2$ receptor antagonists on laryngeal neurophysiology in the rat

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Abstract

Hypophonia is an early symptom in Parkinson’s disease that involves an increase in laryngeal muscle activity, interfering with voice production. Our aim was to use an animal model to better understand the role of different dopamine receptor subtypes in the control of laryngeal neurophysiology. First, we evaluated the combined effects of SCH23390—a D₁ receptor antagonist with a D₂ receptor antagonist (eticlopride) on laryngeal neurophysiology, and then tested the separate effects of selective receptor antagonists. Thyroarytenoid (TA) and gastrocnemius (GN) muscle activity was measured at rest and while stimulating the internal branch of superior laryngeal nerve (iSLN) to elicit the laryngeal adductor response (LAR) in alpha-chloralose anesthetized rats. Paired stimuli at different inter-stimulus intervals between 250 and 5000 ms measured central conditioning of the LAR. Changes in resting muscle activity, response latency, amplitude and LAR conditioning after each drug were compared with the saline control. SCH23390 alone increased the resting TA muscle activity (p < 0.05). With the combined SCH23390 + eticlopride or SCH23390 alone, response latency decreased (p < 0.01), amplitude increased (p < 0.01) and the test LAR was reduced at 2000 ms ISI (p < 0.01). No LAR changes occurred when eticlopride was administrated alone at a low dose and only a tendency to suppress responses was found at a high dose. No changes in GN muscle activity occurred in any of the groups. The results suggest that a loss of stimulation of D₁ receptors plays a significant role in laryngeal pathophysiology in PD.

Key words: Dopamine, Receptor antagonist, Larynx, Sensorimotor reflex
Introduction

In Parkinson’s disease (PD), laryngeal motor control abnormalities frequently occur early in the disorder affecting voice and speech production (Logemann et al. 1978). When laryngeal muscle control was examined prior to treatment early in the disease, increased muscle activity was associated with vocal fold bowing and greater impairment in voice onset and offset control for speech (Gallena et al. 2001). Similar increases in background muscle activity were found in labial muscles that interfered with speech production in untreated patients with PD (Leanderson et al. 1971). In both studies, the abnormally high levels of muscle activity were reduced and speech production improved when the patients were administered a therapeutic dose of levodopa (Leanderson et al. 1971; Gallena et al. 2001).

As the disease progresses, however, levodopa becomes less effective for reducing some symptoms such as speech impairment, abnormal posture, gait and balance (Rascol et al. 2003). Further, deep brain stimulation effects on speech and voice are varied, when compared to benefits on limb control (Dromey et al. 2000; Rascol et al. 2003; Rousseaux et al. 2004). These observations led to the suggestion that the disease mechanisms underlying laryngeal and speech symptoms may differ from those mediating the effects on other motor symptoms (Dromey et al. 2000) and that speech symptoms are less benefitted by levodopa than other motor symptoms (Plowman-Prine et al. 2009). On the other hand, a careful examination of different speech attributes found that some speech symptoms relate to motor symptoms while others do not in persons with PD (Goberman 2005).
A number of motor control characteristics might provide explanations for the possible differences in response to treatment between limb and speech and voice deficits in PD. Speech is a fine motor control task more like handwriting than walking in that it requires precision and skill. However, fine motor control tasks are not necessarily less sensitive to levodopa, as handwriting appears to be highly responsive to dopamine enhancement in PD (Visser et al. 2006). As PD progresses, different effects may alter midline brainstem motor control affecting laryngeal control in PD relative to other brain regions. Speech may show limited benefit from dopamine enhancement similar to other midline functions such as gait, posture, and postural stability (Visser et al. 2006). Midline brainstem motor control regions may be affected earlier by the disease process than other brain regions in PD. Some have proposed a caudal to rostral spread of the disease moving from involvement of the dorsal motor nucleus of the vagus in the brainstem upwards through the medulla, the pontine tegmentum in the midbrain and later reaching the cerebral cortex (Braak et al. 2003). Others have not found support for this in that the substantia nigra was involved in 100% of cases and only half of the cases fit the pattern of caudal to rostral spread (Kalaitzakis et al. 2008).

The effect of dopamine deficiency on laryngeal neurophysiology is of importance for attempting to understand the mechanisms involved in the voice abnormalities in PD and whether these mechanisms are different from those mediating limb motor control abnormalities. Few animal studies have addressed laryngeal pathophysiology in PD partly because the mammalian phonatory system differs considerably from the human speech system (Jurgens 2002). Mammals express innately programmed vocalizations during isolation, pain, and reproductive/sexual functions. These vocalizations are controlled by central pattern generators in the periaqueductal grey and the parvocellular
pontine reticular formation with inputs to laryngeal and respiratory motoneuron pools in the brainstem (Jurgens 2009). Speech in humans, on the other hand, is cortically driven and inputs to the motoneurons in the brainstem descend via rapidly conducting corticobulbar pathways (Jurgens 2002, 2009). Because of the neural control differences between human and animal vocalizations, we chose to assess the effects of dopamine system dysfunction at the final common pathway for both animal vocalizations and voice and speech in humans: that is, the brainstem systems involved in laryngeal muscle control.

Laryngeal sensory stimulation can elicit laryngeal muscle responses, referred to as the laryngeal adductor response (LAR), via a well defined pathway in the brainstem in the cat (Ambalavanar et al. 2004). Further, LAR response conditioning using paired sensory stimuli can quantify central excitation and inhibition in the brainstem as has been done in humans (Ludlow et al. 1995). In the present study, we used LAR conditioning in the rat to investigate changes in central excitation and inhibition in the LAR brainstem system with dopamine receptor antagonism. As the LAR is very similar to the blink reflex (Bhabu et al. 2003), it was predicted that loss of dopamine receptor stimulation might have effects on the LAR similar to those seen with the blink reflex. Studies of blink reflex conditioning in persons with PD have shown a marked loss of inhibition of blink reflex conditioning in humans with PD (Lozza et al. 1997). Based on the previous findings on the blink reflex in PD, we hypothesized that with dopamine depletion the laryngeal adductor response would be hyperexcitable during response conditioning.
As levodopa is non-selective in its action previous studies of the neurophysiological effects of levodopa on laryngeal and labial muscles in persons with early PD (Leanderson et al. 1971; Gallena et al. 2001), did not address the different dopaminergic receptor subtypes. There are two classes of dopamine receptors: D1-like receptors and D2-like receptors (Memo 1990; Seeman et al. 2000). If selective receptor blockers have specific actions within the basal ganglia that alter laryngeal neurophysiology differently, more targeted approaches for addressing patients’ deficits in this system might be indicated. The processes initiated by stimulation of D1 and D2 receptor subtypes may interact in a synergistic way to alter basal ganglia output (Walters et al. 1987; Weick and Walters 1987; Pollack 2004), and may modulate laryngeal neurophysiology differently.

Decreased blink rate is exhibited in PD (Agostino et al. 2008) and both D1 and D2 dopamine receptor agonists increase blink rate in primates (Elsworth et al. 1991) although these two receptor subtypes may interact. For example, D2 receptor activation could inhibit D1-mediated increases in eye blinking (Jutkiewicz and Bergman 2004). Spontaneous blinking, however, differs from blink reflex excitability. Although the blink rate is reduced in PD patients (Agostino et al. 2008), these patients have hyperexcitable blink reflex conditioning (Lozza et al. 1997). We hypothesized that, D1 and D2 dopamine receptor blocking may have different effects on laryngeal muscle activity and LAR conditioning. Such information may increase our understanding of laryngeal neurophysiological deficits in PD and the clinical effects of levodopa on voice and speech function.
Based on the abnormalities in laryngeal muscle control that occur in untreated PD patients, we hypothesized that some selective dopamine receptor antagonists would have an excitatory effect on resting laryngeal muscle activity, which would differ from the effects on limb muscles and that different subtypes of dopamine receptor antagonists would have different effects on laryngeal muscle activity. Further, based on previous findings of blink reflex conditioning abnormalities in PD, we hypothesized that R2 initial conditioning responses would show enhanced excitability of the LAR to sensory input with the administration of the dopamine receptor antagonists, and that R2 test responses would show reduced suppression of the LAR during conditioning following the administration of selective dopamine receptor antagonists.

To examine this in the present study, we measured laryngeal resting muscle activity and the LAR evoked by the iSLN stimulation in the rat. To study the effects of dopamine depletion on laryngeal muscle activity and sensorimotor modulation, we used a combination of SCH23390, a selective D₁ receptor antagonist, and eticlopride, a selective D₂ receptor antagonist. Furthermore, we administered SCH23390 and eticlopride separately to examine the effects of modulating each of these selective dopamine receptor subtypes independently on laryngeal neurophysiology. Here we used selective dopamine receptor antagonists to study the effects of dopamine depletion on laryngeal neurophysiology. Others have proposed using unilateral injection of 6-hydroxydopamine (6-OHDA) or haloperidol to study the effects of dopaminergic depletion on ultrasonic vocalizations in the rat (Ciucci et al. 2007). We chose not to use this model because the laryngeal system is a bilateral midline system and less likely to show deficits following a unilateral 6-OHDA injection than limb control. For example, vocalization calls were not reduced in number or duration despite severe deficits on
contralateral limb movement following unilateral 6-OHDA injections in rats (Ciucci et al. 2009). Further, although levodopa and haloperidol are used clinically to augment or depress the dopamine systems in PD and schizophrenia respectively, neither have selective actions and could affect other neurotransmitters besides dopamine. As changes in limb muscle activity have been induced by dopamine antagonists in rats (Hemsley and Crocker 2001; Hemsley et al. 2002), we measured activity in the gastrocnemius muscle – a limb muscle, to compare the effects of dopamine on both limb and laryngeal muscles simultaneously.

**Materials and Methods**

*Animals and experimental design*

Thirty-seven adult male Sprague-Dawley rats weighing between 250 and 400 g (Harlan, Indianapolis, IN) were maintained on a 12 hour light/dark cycle and given *ad libitum* access to food and water. All procedures were carried out in accordance with National Institute of Health guidelines on the care and use of laboratory animals and the study protocol was approved by the National Institute of Neurological Disorders and Stroke Animal Care and Use Committee. All the drugs were dissolved in 0.25 ml volumes of normal saline for iv injection in all groups. The rats were divided into three groups: group 1 (0.5 mg/kg SCH23390 + 0.5 mg/kg eticlopride, n = 11), group 2 (0.5 mg/kg SCH23390, n = 15) and group 3 (0.5 mg/kg eticlopride, n = 11)... Ten animals had recordings made from both the right and left thyroarytenoid (TA) muscles in each group, however, because the gastrocnemius (GN) was not available in all of these animals, additional animals were added to each group to gather comparable data on the GN muscle to have similar group sizes for the laryngeal and limb muscles. For the SCH23390 + eticlopride, seven animals had both the TA and GN muscles recorded,
three had just TA recordings and an additional animal had only the GN recorded
bringing the total with TA recordings to 10 and the total with GN recordings to eight
animals. For the SCH23390 alone, five animals had both TA and GN recordings, five
had only TA recordings and an additional five animals had the only the GN recorded
bringing the total number with TA recordings to 10 and the total with GN to 10
animals. For eticlopride alone, eight has TA and GN recordings, two had only TA
recordings and an additional animal had GN alone bringing the total with TA
recordings to 10 and the total number with GN recordings to nine animals.

Surgery and electrical stimulation

Each animal was anesthetized with a 3 – 4 % mixture of isoflurane (Sigma, USA) and
100 % oxygen, in an induction chamber then moved to the surgical table and a 16
gauge endotracheal cannula was inserted via the oral cavity for maintenance on 3 %
isoﬂurane. After percutaneous exposure of the trachea, a 14 gauge curved tracheostomy
cannula was inserted at the fifth tracheal ring and connected to the anesthesia machine
(Matrx, NY) with a pressure controlled ventilator (Kent Scientific, CT). The oral
cannula was then removed and the animal was maintained on the ventilator with 3 %
isoﬂurane between 40 – 60 breaths per minute, adjusted by weight, with a 2 ml tidal
volume. An ocular lubricant was placed on the cornea and lidocaine gel into the ear
canals before ear bars were placed and the head was fixed onto a stereotactic frame
(Stoelting, IN) in the supine position. Cardiac and respiratory rates were monitored
continuously by EKG and endotracheal CO₂. Heart rate, respiratory rate, oxygen
saturation and CO₂ levels were recorded every 15 min. The rectal temperature was
maintained at 37 ± 0.5°C with a circulating water heating blanket (Gaymar, NY) to
prevent hypothermia. A tail vein iv provided saline at a rate of 3 ml/kg/hr. Because the
laryngeal response is suppressed by isoflurane, following surgery isoflurane was gradually reduced while a 15 mg/ml alpha-chloralose solution was administered by iv drip between 18 and 36 μl/min, at 340 to 360 mg/kg for 4 hours.

Superior laryngeal nerve (SLN) stimulation and muscle recordings

The right/left SLN was exposed and positioned over a hooked bipolar platinum stimulating electrode (FHC Inc., Bowdoinham, ME) with 1.75 mm coated diameter, and 0.8 cm spacing between the two poles and connected to a stimulus isolator (A365, WPI). The nerve stimulation sites were immersed in warm mineral oil.

For electromyographic (EMG) recordings, two Teflon-coated 2 stainless wires (0.011 mm coated diameter) with 1 mm bared tips contained in a 27 gauge needle were inserted through the cricothyroid space into the TA muscle on each side. The same type of electrode was inserted into the gastrocnemius (GN) muscle of each hind leg. All four muscles, the left and right TA and GN muscles, were recorded throughout the study on a computer (AD instruments).

After sectioning the external branch of the SLN to prevent cricothyroid muscle contraction from interfering with TA muscle recordings animals were stabilized for 20 minutes, Electrical stimulation of the iSLN on one side began at 10 μA with a pulse width of 20 ms and was increased until the threshold level for eliciting a reliable laryngeal adductor R2 response was determined (Fig. 1). The LAR responses in the rat were comprised of an ipsilateral short latency R1 (7.35 ± 0.26 ms) and a bilateral long latency R2 response (22.20 ± 2.08 ms) of the TA muscles. In this study, R1 responses occurred in only three animals and were irregular. Therefore, only the consistent R2
responses were recorded and analyzed. The stimulus intensity was then set at three times the stimulation threshold for eliciting R2 responses and the same level was used throughout the experiment. Between 5 and 6 pairs of conditioning stimuli each followed by a test stimuli were administered at each inter-stimulus interval (ISI) of 250, 500, 1000, 2000 and 5000 ms. The R2 response to the first stimulus (the conditioning stimulus in a pair) was the conditioning response and the response to the second stimulus (the test stimulus) was the test response (Fig. 1). A pilot study confirmed that complete suppression of test R2 responses occurred at intervals of 500 ms or less while test response amplitudes increased at 2000 ms ISI. At least 40 s occurred between stimulus pairs to avoid habitation. The stimulation rate was programmed using Master-8 (A.W.P.I., Israel). The EMG signals were amplified (Grass Telefactor, Model RPS312RM, RI), filtered between 30 Hz and 10 kHz, monitored on-line with a digital oscilloscope (Tektronix TDS 420, OR) and digitized at 20k Hz with Chart 5 for Windows (AD Instruments, Inc) for off-line analysis. The mean of the conditioning and test responses were computed for each set at each ISI using Matlab customized software before statistical analysis.

Drug administration

The first part of the study measured changes with the administration of saline in resting muscle activity and the response latency and amplitude to the conditioning stimuli and the response characteristics after the test stimuli as the control condition. Initially, the laryngeal responses were recorded in each animal before saline infusion and included 5 to 6 trials at each ISI of 250, 500, 1000, 2000, and 5000 ms. Animals were then maintained under quiet conditions for up to 30 minutes. After a 0.25 ml bolus saline solution was administered by iv, another set of laryngeal responses were recorded.
beginning 5 minutes later (post-saline). Following the second stimulation set, the animals were maintained under quiet conditions for another 30 minutes and then a third set of laryngeal responses (pre-drug) was recorded at 30 minutes post saline infusion. After 30 minutes of further stabilization, a 0.25 ml volume bolus containing either the mixture of SCH23390 (0.5 mg/kg) + eticlopride (0.5 mg/kg) (Sigma, MO), SCH23390 (0.5 mg/kg) or eticlopride (0.5 mg/kg) was administered by iv and a fourth set of laryngeal responses was recorded 5 minutes later (5 min post-drug). Finally, one hour following drug administration, a fifth set of laryngeal responses was recorded (1 hr post-drug).

At the end of each experiment, the recording wires were cut from the outside leaving the tips of the wires inside the laryngeal muscles, animals were administered an overdose of propofol (10 mg/ml, iv) and the larynx was dissected to confirm the position of the recording wires in the TA muscles. Only data collected from animals with confirmed electrode placement in the TA muscles were analyzed.

Data analysis

EMG signals were digitized at 20k samples per second with anti-aliasing filtering at 10k Hz, stored on a computer, rectified and visually analyzed off-line using an interactive software program written in Matlab R2006a allowing the operator to mark the onset and offset of each laryngeal R2 response. After marking, the program computed the response latency, duration, and the integrated area under the curve from the rectified TA EMG response. For each response, the mean baseline activity during a 20 ms interval before the first stimulus onset was computed and multiplied by the duration of the following response before being subtracted from the total area under the
curve of the response to correct for any changes that may have occurred due to changes in muscle activity. The corrected integrated amplitude was computed as follows:

Corrected Integrated Amplitude = Integrated Amplitude of Response – (Mean Baseline × Duration of the Response)

All responses were reviewed and confirmed by two members of the research team blinded to animal and conditions. In instances where no response was observed, an interval of 5 ms was marked as the response. When the baseline activity × response duration was then subtracted, the response area under the curve was close to zero. For each condition, the following measures were made: the mean level of baseline activity, the latency of a response to the stimulus, and the total area under the curve of a response in microvolt-milliseconds after subtracting the corresponding baseline activity. The conditioning R2 responses for an animal were averaged over 25 – 30 trials (5 – 6 per interval for each of the ISIs) to compute mean ± standard deviation (SD) of R2 responses on the ipsilateral and contralateral sides to iSLN stimulation. The responses from both sides were averaged for analysis after finding no differences between the R2 responses in the ipsilateral and the contralateral sides.

To compare the percent change of pre-post saline with the percent change of pre-post drug for the resting muscle activity, response latency and response amplitude of the conditioning R2 responses, the percent change between pre and post measures after saline and after drug were computed as:

Percent Change of conditioning responses (%) = [(Post – Pre) / Pre] × 100
To measure the percent change in a test response from a conditioning response at each ISI, we computed:

\[
\text{Percent Change (\%) = \left(\frac{\text{Integrated Amplitude of test response} - \text{Integrated Amplitude of conditioning response}}{\text{Integrated Amplitude of conditioning response}}\right) \times 100}
\]

The mean percent change (computed over 5-6 trials) between pre and post saline and between pre and post drug was then computed for each ISI for each animal in each condition.

Statistical analyses

One-way repeated ANOVAs were used to compare different conditions within the same group of animals including:

1) the change in resting activity in the TA muscle post saline with the change post SCH23390 + eticlopride and similarly the change post SCH23390 with post saline and the change post eticlopride alone with post saline (p < 0.05); and

2) the change in LAR conditioning response latency and amplitude post saline with the change post SCH23390 + eticlopride and similarly the change post SCH23390 with post saline and the change post eticlopride alone with post saline (using a Bonferroni corrected p ≤ 0.025).

3) the test response percent change from the conditioning LAR response amplitude before and after drug while testing for interaction effects with ISIs for SCH23390 + eticlopride, SCH23390 alone and eticlopride alone. If before
versus after drug effects or interactions with ISI were significant (p ≤ 0.05), post hoc Wilcoxon signed rank tests were used to compare before versus after drug effects at each ISI.

Kruskal–Wallis comparisons were conducted between changes in resting activity in the TA with the change in resting activity of the GN post saline with post SCH23390 + eticlopride and similarly, the change in the two muscles post saline were compared with post SCH23390 alone and with post eticlopride alone (p ≤ 0.05).

One–way ANOVAs between animal groups were used to compare the effects of the two dopamine receptor antagonists:

1) on changes in resting muscle activity post saline with change post each antagonist

2) on changes in latency and amplitude of LAR responses to conditioning stimuli post saline with change post each antagonist (p <0.025);

One-way ANOVAs between groups were used to determine whether or not eticlopride modified the effect of SCH23390 on muscle activity, response latency and integrated conditioning response amplitude between SCH23390 + eticlopride and SCH23390 alone using a Bonferroni corrected p value of 0.0167 for statistical significance.

Finally, to examine whether or not any changes in test responses with antagonists were secondary to increases in the amplitude of the conditioning response with antagonists, we computed r values between the percent increase in amplitude in the conditioning response post antagonist in relation to the change in percent conditioning on the test.
response at ISIs where there were significant changes in conditioning effects with the antagonist ($p \leq 0.05$).

Results

Effects of dopamine receptor antagonists on resting TA muscle activity

No significant change in the resting TA muscle activity occurred 5 min ($F = 2.222; p = 0.174$) and 1 hr ($F = 0.145; p = 0.714$) post SCH23390 + eticlopride injection (Fig. 2A). When the effects of SCH23390 alone were compared with saline 5 min post drug, there was a significant increase in muscle activity ($F = 5.986; p = 0.037$) which did not persist at 1 hr post drug ($F = 1.725; p = 0.225$) (Fig. 2B). No effects on resting TA muscle activity was found in eticlopride alone ($F = 0.089; p = 0.773$; Fig. 2C). Further, no differences were found between the effects of SCH23390 + eticlopride and SCH23390 alone on resting TA muscle activity ($F = 0.132; p = 0.721$).

Comparison of the effects of dopamine receptor antagonists on resting TA and GN muscle activity

No significant changes in GN resting muscle activity were found following SCH23390 + eticlopride ($F = 0.117; n = 8, p = 0.743$, Fig. 2D). No significant changes in GN muscle activity were found following either SCH23390 ($F = 0.535; n = 10, p = 0.483$, Fig. 2E) or eticlopride alone ($F = 0.110; n = 9, p = 0.750$, Fig. 2F).

Comparisons between the effects of drug on TA versus GN were between groups because some of the animals were different in the muscles recorded. Kruskal Wallis group comparisons were used because of group differences in the standard deviations. For SCH23390 + eticlopride there were no differences between effects in muscle
activity between the TA and GN (Mann Whitney $U = 24; p = 0.155$). For SCH23390 alone no significant difference was found between muscles (Mann Whitney $U = 21; p = 0.028$), and for eticlopride alone there was no difference in change in activity between the TA and GN muscles (Mann Whitney $U = 33; p = 0.327$). A typical recording of increased resting muscle activity in TA (increased motor unit firing) but not in GN muscles following SCH23390 (0.5 mg/kg, iv) infusion alone is shown in Figure 3.

**Comparison of the effects of $D_1$ and $D_2$ antagonists on resting muscle activity**

A significant difference was found between animals receiving SCH23900 and those receiving eticlopride ($F = 4.545; p = 0.047$) with the mean percent difference following SCH23390 = 5.242 ± SD = 4.704 and the mean percent difference following eticlopride = 0.968 ± SD = 4.249.

**Effects of dopamine receptor antagonists on the conditioning laryngeal R2 responses**

For SCH23390 + eticlopride administration, conditioning R2 responses were reduced in latency ($F = 11.167; p = 0.01$) from 23.18 ± SD 1.41 ms to 19.64 ± SD 1.00 ms and increased in amplitude ($F = 66.362; p < 0.0001$) at 5 min post. At 1 hr post SCH23390 + eticlopride administration, the amplitude remained increased ($F = 15.708; p = 0.004$) but the response latency returned to pre-drug level at 22.57 ± SD 1.69 ms ($F = 0.249; p = 0.631$, Fig. 4).

For SCH23390 administration alone, a similar trend to SCH23390 + eticlopride for a reduced latency from 24.23 ± SD 1.17 ms to 19.33 ± SD 0.93 ms ($F = 62.792; p < 0.001$) and increased amplitude ($F = 12.187; p = 0.007$, Fig. 5) 5 min post administration. At 1 hr post SCH23390 administration, the latency continued to be
decreased around 21.34 ± SD 1.20 ms ($F = 20.031; p = 0.002$) while the amplitude still showed a trend towards increasing ($F = 5.529; p = 0.047$).

No changes were found at 5 min or 1 hr post eticlopride administration in both the latency ($F = 2.643, p = 0.138$; $F = 2.744, p = 0.132$) and the amplitude ($F = 2.388, p = 0.157$; $F = 0.234, p = 0.640$) (Fig. 6).

No differences were found between the combined SCH23390 + eticlopride compared with SCH23390 alone on latency ($F = 3.004, p = 0.100$) and integrated amplitude ($F = 0.846, p = 0.370$) of the conditioning R2 responses 5 min after administration.

**Effects of dopamine antagonists on the conditioning changes in laryngeal R2 responses**

The combination of SCH23390 + eticlopride had a significant effect ($F = 6.778; p = 0.029$) on conditioning changes in laryngeal R2 test responses. Post hoc Wilcoxon signed rank test indicated a significant increase in conditioning suppression at 250 ms ISI ($Z = -2.497; p = 0.013$) and 500 ms ISI ($Z = -2.293; p = 0.022$) and reduction in conditioning facilitation at 2000 ms ISI ($Z = -2.599; p = 0.009$, Fig. 7A). The percent change in test R2 responses between the pre- and 5 min post drug at each of the ISIs also showed a significant change in conditioning effects with SCH23390 ($F = 10.602; p = 0.01$) and a significant interaction with ISI ($F = 4.068; p = 0.008$). Post hoc testing found a decrease in conditioning facilitation at 2000 ms ISI ($Z = -2.395; p = 0.017$, Fig. 7B). No effects of eticlopride were found on conditioning ($F = 0.114; p = 0.743$, Fig. 7C).
We tested whether the conditioning changes in test responses could be related to increases in the conditioning responses with the antagonist. For SCH23390 + eticlopride, no relationship with change in amplitude of the conditioning R2 response was found at 250 ISI ($r = 0.225; p = 0.532$), at 500 ms ISI ($r = 0.452; p = 0.190$) or at 2000 ISI ($r = 0.052; p = 0.887$). This suggested that the significant increase in conditioning suppression at 250 ms and 500 ms ISI and the significant reduction in facilitation at 2000 ms ISI were not related to the increase in the conditioning response amplitude post SCH23390 + eticlopride.

For SCH23390 alone, a significant relationship was found between the conditioning R2 response amplitude and the reduced facilitation of the test response at 2000 ISI ($r = -0.688; p = 0.028$). This suggested that the greatest reduction in facilitation at 2000 ms ISI occurred when there was a greater percent increase in muscle activity in response to the conditioning stimulus post SCH23390.

The lack of effects of eticlopride (0.5 mg/kg) on the LAR was further examined by increasing the dosage from 0.5 to 1 mg/kg in 4 animals. Because only 4 animals were studied, no statistical analyses were conducted.

Effects of high dose eticlopride on the resting TA and GN muscles activity

Resting muscle activity was not changed in either the TA or the GN muscles with the higher dosage of eticlopride (Fig. 8A, B). Similar results were found at 5 min and 1 hr post the high dose eticlopride.

Effects of high dose eticlopride on the conditioning laryngeal R2 responses
Conditioning R2 responses 5 min post 1 mg/kg eticlopride administration showed a tendency to increase in latency (Fig. 8C) and decrease in amplitude (Fig. 8D). At 1 hr post eticlopride administration the amplitude tended to remain decreased and the response latency remained somewhat increased.

Effects of high dose eticlopride on the laryngeal R2 test responses

No marked changes were seen in the % change in the test responses post high dose eticlopride (Fig. 8E).

Discussion

The data demonstrate that D1 and D2 dopamine receptor antagonists have different effects on the regulation of the laryngeal neurophysiology and sensorimotor responses in rats.

Changes in resting muscle activity with dopamine receptor blockade

Comparisons of changes in resting muscle activity with the administration of D1 and D2 receptor antagonists showed increases in laryngeal muscle activity only with administration of the D1 receptor antagonist. Further, the effects of the selective D1 and D2 receptor antagonists showed different effects on laryngeal muscle activity and neither altered limb muscle activity. These results may indicate that increases in laryngeal muscle activity found in persons with PD may have been due to the loss of dopamine binding at D1 receptors (Gallena et al. 2001). Other studies have shown dopamine D1 and D2 receptors in the ventral striatum, and D1 receptors in the substantia nigra (SN) regulated limb muscle activity in awake rats (Hemsley and Crocker 2001). A non-selective dopamine receptor antagonist which blocks both D1 and D2 receptors
administered either centrally or systemically can increase the limb muscle activity in
awake rats (Double and Crocker 1995; Hemsley and Crocker 1998). However, in this
study only the D1 antagonist alone was effective while the D2 antagonist and the
combined D1 and D2 antagonists did not change the laryngeal muscle activity. The
results, however, did not suggest that these dopamine receptor subtypes may modulate
muscle activity differently as there was no difference between the effects of the two
antagonists combined versus the D1 antagonist alone.

On the other hand, the effects of the D1 receptor subtype on the laryngeal and limb
muscles differed although we cannot discount the potential role of alpha-chloralose
anesthesia used in this study while the studies in the limb muscles were performed in
awake animals (Hemsley and Crocker 2001). Alpha-chloralose anesthesia induces a
chemical restraint without altering autonomic reflex activity or myocardial function.
The laryngeal muscles are part of the respiratory system and are active at rest (Bartlett
et al. 1973; Kuna et al. 1988; Kuna et al. 1994), while the GN is quiet in a supine
animal under anesthesia. The D1 receptor antagonist increased the muscle activity in the
laryngeal but not the limb muscles. Differences in the resting levels of motor neuron
firing in the two muscle systems under alpha-chloralose anesthesia might explain these
results. We maintained our animals at level 3 anesthesia in this study. At this stage,
breathing becomes shallow and the animal no longer responds to foot pinch although
respiratory related laryngeal muscle activity is present (Whelan and Flecknell 1992;
McKelvey and Hollingshead 2000). We used tracheal ventilation to modulate the
respiratory rate and pressure close to the natural rhythm and maintained normal
respiratory activity in the laryngeal muscles. If ventilation was stopped, the animal's
respiratory rhythm reappeared. However, limb muscle activity was absent throughout
the experiment. This may account for the lack of modulation of the GN muscles by dopamine receptor antagonists under anesthesia. Whether dopamine receptor antagonists differentially modulate the spinal and brainstem respiratory systems, or exert different effects on laryngeal and limb muscles under the alpha-chloralose anesthesia is not clear.

**Dopamine receptor blockade and laryngeal sensorimotor responses**

We postulated that D₁ and D₂ receptor antagonists would enhance the excitability of laryngeal R2 muscle responses to conditioning sensory stimuli. Both the combined D₁ and D₂ receptor antagonists as well as the D₁ receptor antagonist alone increased the amplitude and decreased the latency of laryngeal R2 responses to conditioning stimuli supporting our hypothesis. However, neither the low nor the high dosage of the D₂ receptor antagonist had significant effects on laryngeal R2 responses, and no differences were found between the combined D₁ and D₂ receptor antagonists and the D₁ receptor antagonist alone. Therefore, the results only support an active effect of the D₁ receptor blocker on the laryngeal adductor response.

D₁ and D₂ receptors are the most heavily expressed in the striatal part of the basal ganglia and mediate the responses of striatal neurons to dopaminergic input from the midbrain (Bouthenet et al. 1991; Civelli et al. 1991; Van Tol et al. 1991). Although striato-GPi/SN neurons express D₁ and D₂ receptors the majority mainly express D₁ while the vast majority of striato-GPe neurons express D₂ receptors (Deng et al. 2006). Dopamine is thought to modulate locomotion through activation of different dopamine receptors in the direct and/or indirect pathways. A recent study supports the notion that dopamine receptors are segregated on striatal projection neurons (Gantois et al. 2007).
Some neurons in the dopamine pathways co-express D₁ and D₂ receptors which may have opposing, synergistic or independent effects. Here, we showed only the excitatory effects of D₁ receptor antagonism on the laryngeal responses, and no interaction between the two antagonists. These data strongly suggested that only the D₁ receptors have effects on modulating laryngeal response control in the brainstem.

Previously D₁ and D₂ receptor modulation altered the blink reflex differently across studies. In one study both D₁ and D₂ agonists enhanced blink rate independently in primates (Elsworth et al. 1991) while in another study a D₂ agonist attenuated blinking and a D₁ agonist induced increased in eye blinking (Jutkiewicz and Bergman 2004). The present results indicate that D₁ receptor activation may normally suppress laryngeal sensorimotor responses. This may suggest that the effect of D₁ receptor activation may have a somewhat different effect on the LAR than on the blink rate.

However drug effects may differ between the blink rate and the blink reflex. A study of the effect of haloperidol induced dopamine antagonism on the blink reflex in healthy volunteers found no effect on R2 latencies or amplitude but that the amplitude of the R₁ blink response was enhanced with dopamine antagonism (Raffaele et al. 1988). Because of the many differences between these two studies (only R2 was elicited for the LAR in the anesthetized rat) it is difficult to know whether the effects of D₁ antagonism on the LAR and the blink reflex are similar or not. Both the LAR and the blink are defensive cranial reflexes with similar short (R₁) and long latency (R₂) components. These results suggest that different mechanisms may modulate the LAR and blink reflexes with dopamine antagonism. However, these differences could also be due to possible interactions with the alpha-chloralose anesthesia used in this study.
Dopamine receptors and conditioned laryngeal responses

We found that conditioning suppression increased at 250 and 500 ms when D₁ and D₂ receptor antagonists were combined. Neither of these changes could be explained by increases in amplitude of the conditioning R2 response. On the other hand, facilitation was reduced at 2000 ms ISI with the combined D₁ and D₂ receptors antagonists. This also occurred with the D₁ receptor antagonist alone, which was related to the percent increase in the conditioning response amplitude with D₁ receptor antagonism. Here the method of computation may have contributed to the finding of decreased facilitation at 2000 ms ISI with D₁ receptor antagonism alone.

We can conclude, therefore, that the increased conditioned suppression of the test LAR responses with D₁ + D₂ receptor antagonists in the brain demonstrate increased suppression of laryngeal responses to sensory stimuli with repeated stimulation. These effects of dopamine neurotransmission antagonism may underlie the reduction in upper airway responses such as cough in PD. In the late stages of PD, some patients have reduced cough reflex sensitivity (Ebihara et al. 2003).

The increased suppression with conditioning of the LAR in the rat with D₁ + D₂ antagonism at ISIs below 1000 ms also differs from facilitation of the conditioned blink reflex found contralateral to a complete 6-OHDA lesion in the rat (Basso et al. 1993). Similarly, in PD patients, less suppression of test R2 responses was seen in the R2 blink reflex with conditioning (Kimura 1973) at ISIs between 50 and 600 ms (Lozza et al. 1997). The methods for the studies of the blink and this study of the LAR were not comparable; a unilateral 6-OHDA lesion likely has different effects from systemic
selective receptor antagonists as were used here. Further the effects of reduced
dopamine neurotransmission on conditioning effects on the LAR have not been studied
in patients with PD as was done with the blink reflex (Kimura 1973; Lozza et al. 1997).
As proposed earlier, the LAR and the blink may have different mechanisms involved; a
comparison of the two in the same animal model or in PD patients is needed to
determine if the physiological effects of reductions in dopamine neurotransmission on
the blink and the LAR differ.

We cannot generalize our findings in a rat model of selective receptor antagonists to
voice disorders in PD patients. However, abnormally high levels of muscle activity in
the laryngeal and labial muscles have been observed in untreated patients with PD
(Leanderson et al. 1971; Gallena et al. 2001; Zarzur et al. 2007). In PD patients treated
with clinically effective levels of levodopa, the firing rate of the TA motor units and
labial muscle activity are both decreased (Leanderson et al. 1971; Luschei et al. 1999;
Gallena et al. 2001). The reduction in TA activity in PD patients treated to effect with
levodopa later in the disease course may also reflect sensory gating abnormalities
occurring in the more severe form (Schneider et al. 1986; Jobst et al. 1997). Levodopa
is the most commonly used medication for PD but others have proposed that it may not
be as effective for speech disorders as for limb control in PD patients (Wolfe et al.
1975). Similarly, no significant benefits of apomorphine on voice and speech
articulation were found in comparison with placebo control (Kompoliti et al. 2000).
Both levodopa and apomorphine are non-selective dopamine agonists in the brain with
a higher affinity for the D2 receptor in the brain (Jenner 2002). No studies have
explored the effects of selective D1 or D2 agonists on laryngeal physiology in PD
patients. The depletion of dopamine in PD patients that affects the laryngeal
sensorimotor modulation may be via different dopamine receptors and/or different neural pathways from limb motor control (Jurgens and Ehrenreich 2007). In addition, D₁-like receptors are further divided into D₁A, D₁B and D₅ and D₂-like receptors are divided into D₂S and D₂L, D₂ and D₃ and D₄ receptor subtypes (Memo 1990; Seeman et al. 2000). Our study found a higher level of resting laryngeal muscle activity, increased LAR R2 responses and decreased facilitation in a D₁ dopamine receptor antagonist induced rat model. This may suggest that loss of D₁ receptors may play an important role in laryngeal abnormalities in PD. However, further studies are needed to explore the effects of the specific subtypes of dopamine receptors on laryngeal physiology to determine the mechanisms of dopamine neurotransmission on the cranial musculature involved in laryngeal functions in dopamine depleted animal models. Studies in animals may provide the bases for exploring the potential of future selective dopamine enhancement therapies for voice deficits in PD in humans.

Conclusion

We conclude that reduced neurotransmission via the D₁ receptor subtypes serves to increase resting muscle activity similar to that seen in early untreated PD. Further, laryngeal adductor responses to afferent input have a shorter latency, increased amplitudes and reduced excitation of test responses after reductions in D₁ neurotransmission. D₁ dopamine receptors may modulate the control of laryngeal responses to sensory inputs in the brainstem, and may affect laryngeal neurophysiology differently from limb movements. Further studies are needed to address these issues in humans.

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Legends

Fig 1. Conditioning and test responses recorded in the TA muscle. Examples are shown of conditioning and test R2 responses at each inter-stimulus interval (250, 500, 1000, 2000, 5000 ms ISI). Test responses were suppressed at 250, 500 and 1000 ms ISIs and increased at 2000 ms ISI relative to conditioning responses. The baseline (the resting muscle activity before the iSLN stimulus) was measured for 20 ms before stimulation onset.

Fig 2. Box plots of percent change from resting activity of TA and GN muscles in response to SCH23390 + eticlopride, SCH23390 and eticlopride. No statistically significant increase of resting TA muscle activity was found in SCH23390 + eticlopride (A, n = 10) or eticlopride (C, n=10) alone. Resting TA muscle activity increased 5 min post administration of SCH23390 alone (B) (*p = 0.037, n = 10). Neither SCH23390 + eticlopride (D, n = 8), SCH23390 (E, n = 10) nor eticlopride (F, n = 9) had effects on the resting GN muscle activity.

Fig 3. Effects of SCH23390 on the resting TA and GN muscle activity. A representative recording of EMG activity shows increased resting TA muscle activity (top trace) but no change in resting GN muscle activity (second trace) post SCH23390 administration. In the top trace, the arrows mark the time points for the start and end of the iv injection which took less than 5 seconds. Resting TA muscle activity increased before the end of the injection. The bottom two traces, with an expanded view of the first 10 seconds following injection, show increased motor unit firing in the TA post SCH23390 administration with no resting activity change in the GN muscle.
Fig 4. Box plots of percent change from effects of SCH23390 + eticlopride on the latency and amplitude of the laryngeal conditioning R2 response. The top traces showing representative EMG recording of conditioning R2 responses changes induced by SCH23390 + eticlopride compared to the saline. Latency of conditioning R2 response decreased and amplitude increased post SCH23390 + eticlopride administration. Percent changes of the response latency decreased and amplitude increased at 5 min after administration and the latency returned to pre-drug level and amplitude remained increasing 1 hr after SCH23390 + eticlopride administration compared with the saline control (*p ≤ 0.01, n = 10).

Fig 5. Box plots of percent change from effects of SCH23390 alone on the latency and amplitude of laryngeal conditioning R2 responses. The top traces showing representative EMG recording of conditioning R2 responses changes induced by SCH23390 alone compared to the saline. Latency of conditioning R2 response decreased and amplitude increased after SCH23390 administration. Percent changes of the response latency decreased at 5 min post SCH23390 administration and the latency maintained decreasing 1 hr post SCH23390 compared with the saline control (*p ≤ 0.01, n = 10).

Fig 6. Box plots of percent change from effects of eticlopride (0.5 mg/kg) alone on the latency and amplitude of the laryngeal conditioning R2 response. The top traces showing representative EMG recording of conditioning R2 responses changes induced by eticlopride alone compared to the saline. No effects of eticlopride (0.5 mg/kg) were
found on the latency and amplitude of conditioning R2 response at 5 min and 1 hr after eticlopride administration compared with the saline control (n = 10).

Fig 7. Percent change of test R2 responses induced by SCH23390 + eticlopride, SCH23390 and eticlopride. Conditioning facilitation was decreased by either the combination of SCH23390 + eticlopride (A, *p < 0.05, n = 10) or the SCH23390 alone (B, *p < 0.05, n = 10) at 2000 ms ISI. Conditioning suppression was increased by the SCH23390 + eticlopride at 250 and 500 ms ISIs (B). No effects on conditioning changes were found in eticlopride administration group (C) at any ISIs (n = 10).

Fig 8. Effects of high dose eticlopride (1 mg/kg) on baseline activity of TA and GN muscles, the latency, amplitude of the laryngeal conditioning R2 response and conditioning changes in the test R2 response.

No effects of high dose eticlopride were found on the resting muscle activity of TA (A) and GN (B) muscles. Conditioning R2 response latency (C) showed a tendency to increase and amplitude (D) decrease 5 min post administration. The amplitude tended to remain decreased and the latency remained somewhat increased 1 hr later. No effects of high dose eticlopride were found on the conditioning changes in the test R2 response at each ISI (E) (n = 4).
A. **SCH23390 + Eticlopride**

% Change of Test R2 Response vs. ms

- Pre-SCH23390 + Eticlopride
- Post-SCH23390 + Eticlopride

B. **SCH23390**

% Change of Test R2 Response vs. ms

- Pre-SCH23390
- Post-SCH23390

C. **Eticlopride**

% Change of Test R2 Response vs. ms

- Pre-Eticlopride
- Post-Eticlopride