**Responses of Adenohypophyseal Hormones to Substance P Administration in Geldings:
Comparison to Responses after Brief Exercise and Sulpiride Administration**

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ABSTRACT

Nine adult geldings were used in three experiments to study the possible role of substance P in the prolactin responses to non-dopaminergic stimuli. Experiment 1 was performed as an incomplete Latin square design to determine the secretory responses of prolactin, growth hormone (GH), adrenocorticotrophic hormone (ACTH), thyroid stimulating hormone (TSH), luteinizing hormone (LH) and follicle stimulating hormone (FSH) to intravenous administration of substance P. Doses tested and compared to no peptide (0 dose, control) were 62, 125, 250, and 500 ug of substance P. The three highest doses of peptide caused an immediate rise in heart rate, sweating, salivation, rhinorrhea, stretching of hind legs, and defecation. The lowest dose (62 ug) caused minor sweating, some rhinorrhea, and a rise in heart rate. Recovery from these physical responses was complete in approximately 30 min. All doses of substance P caused an immediate rise ($P < 0.01$) in plasma prolactin concentrations, with the three highest doses producing similar responses, and the 62 ug dose producing a minimal response ($P < 0.05$). Concentrations of ACTH ($P < 0.01$) and GH ($P = 0.05$) also increased after substance P administration; concentrations of LH, FSH, and TSH were unaffected. Experiment 2 compared the effects of brief exercise on hormonal characteristics. Two minutes of trotting increased ($P < 0.01$) plasma concentrations of GH, ACTH, and prolactin, as well as LH ($P = 0.055$). Experiment 3 determined the relative responses of prolactin to a fixed dose of sulpiride (0.1 mg/kg of body weight). In general, the prolactin responses to substance P were similar to those after exercise, which were both generally less than after sulpiride. These data are consistent with a possible role of substance P in the prolactin response to stressful stimuli.

1. Introduction

Prolactin is one of several hormones that are rapidly released in response to acute stressors (brief exercise, twitching, physical excitement) in horses [1-3]. Hypothalamic dopamine from the tuberoinfundibular neurons is the main known regulator of prolactin secretion in most, if not all, mammals, including the horse, and interruption of this dopaminergic input to the adenohypophysis via dopaminergic receptor antagonists, such as sulpiride or domperidone, results in an immediate release of prolactin into the peripheral blood [reviewed by [4]]. Whether the prolactin response to stressors in the horse is caused by an interruption (reduction, cessation?) of the **input of the tuberoinfundibular dopaminergic neurons** to the lactotropes, or due to some other mechanism, is currently unknown. One possible alternate mechanism for prolactin release is direct neural input to the adenohypophysis. Innervation of the glandular adenohypophyseal tissue has been reported for rats, monkeys, dogs, and humans [5-8]. In all these species, one neuropeptide that has been consistently associated with this neural input is substance P. Substance P is an 11-amino acid peptide that has been implicated in various physiologic responses, including vomiting, cardiovascular tone, salivary secretion, defensive behavior, vasodilation, and smooth muscle contractions [9-11].

The initial objective of the present experiment was to determine the effect(s) of intravenous administration of substance P on plasma prolactin concentrations in geldings, given its presence as a neurotransmitter in the adenohypophysis in other species. Given the numerous overt physiologic effects of substance P observed in the performance of the experiment, the investigation was expanded to include all anterior lobe hormones. Based on those results, which indicated a generalized stress-like response, a second experiment was conducted to determine the effects of a brief (2-min) exercise bout on the same hormones so that similarities and contrasts

could be assessed. A third brief experiment detailed the prolactin response to a fixed dose of sulpiride for comparison to the prolactin responses to substance P and exercise.

2. Materials and Methods

Nine light horse geldings (castrated for at least 5 years) between the ages of 6 and 22 years were used. They weighed between 450 and 650 kg and had body condition scores between 6 and 8. They were maintained outdoors on native grass pastures in the warm seasons and on winter ryegrass when available. Between seasons, they were supplemented as needed with hay made from the native grasses. These experiments were performed in July and early August of 2015.

2.1. Experiment 1. Substance P Administration

Substance P amide acetate salt was purchased from Bachem Americas, Torrance, CA (catalog item H-1890). It was diluted in 0.155 M sterile saline for injection. Four doses, 500, 250, 125, and 62.5 µg of peptide, plus a sterile saline (control) injection, were administered intravenously. The substance P was diluted to a concentration of 200 µg/mL, thus the four substance P doses were 2.5, 1.25, 0.625, and 0.31 mL; control saline was injected at 2.5 mL. Substance P and vehicle treatments were administered on four different occasions (July 14, 17, 20, and 28, 2015). The first day included doses of 500 and 250 µg as well as saline control. Subsequent days were used to test lower doses (125 and 62.5 µg) and to increase the number of animals getting the higher doses. Final numbers for the saline controls and the 500, 250, 125, and 62.5 µg doses were 6, 7, 9, 8, and 5 geldings, respectively. No gelding received any given dose more than once.

On each morning of treatment, geldings were walked from a small pasture adjacent to the test area into an outdoor chute for restraint. A pre-treatment blood sample (time 0) was collected by jugular venipuncture via a 21-gauge needle into an evacuated 7-mL tube containing 68 USP units sodium heparin (Vacutainer, B-D, Franklin Lakes, NJ). Substance P or saline was then injected intravenously into the same jugular vein, and post-treatment blood samples were collected at 10, 20, 30, 45, and 60 min. All blood samples were placed at 5°C until centrifugation at 1200 x g for 15 min. Plasma was harvested and stored in plastic 12 x 75-mm tubes at -15°C.

External signs of response to the substance P injections were noted on each day, but exact quantification was not attempted. The responses described in the Results section were generally observed for all geldings receiving the 250 and 500 µg doses. Measurements such as heart and respiration rates that required time to assess were performed on a representative sampling of geldings but not all geldings due to manpower restrictions.

Concentrations of prolactin [12], growth hormone (GH [13]), luteinizing hormone (LH [14]), follicle stimulating hormone (FSH [15]), and thyroid stimulating hormone (TSH [16]) were assessed in plasma samples by radioimmunoassays previously validated for horses plasma. Concentrations of adrenocorticotrophic hormone (ACTH) in plasma were measured by radioimmunoassay with commercial reagents (MP Biomedicals, Santa Ana, CA). The intra- and interassay coefficients of variation and limits of detection (sensitivity) of the assays were 7%, 12%, and 0.2 ng/mL for prolactin; 8%, 11%, and 0.5 ng/mL for GH; 6%, 9%, and 0.2 ng/mL for LH; 7%, 11%, and 1.4 ng/mL for FSH; 5%, 8%, and 0.1 ng/mL for TSH; and 6%, 9%, and 8 pg/mL for ACTH. Prolactin was measured in all plasma samples, whereas the other hormones were only measured in the samples collected after treatment with the 250 µg dose of substance P (which included all geldings).

Data for plasma prolactin concentrations were analyzed with PC-SAS (SAS Instit., Cary NC) by analysis of variance (ANOVA) that took into account that the majority of geldings received multiple doses of substance P (incomplete Latin square design) as well as the repetitive nature of the blood sampling (repeated measures[17]). The main effects were dose of substance P, gelding, and day. The three-way interaction, with the main effects pulled out, served as the error for the main effects. The effect of minute of sampling and its interaction with dose were tested with residual error as the error term. Due to a heterogeneity of variance among the treatment doses, two data transformations were assessed for comparison: 1) the ANOVA described above was also performed on the data transformed to the natural log; means derived from the natural log ANOVA were transformed back via exponentiation, and 2) for each treatment occasion for each gelding, the pre-treatment prolactin concentration was subtracted from all the post-treatment prolactin concentrations to obtain residuals; the residuals were analyzed in separate ANOVA for the 500, 250, and 125 μ g challenges (together) and the 62.5 and 0 doses (together).

Data for all other hormonal concentrations (for the 250 μ g dose only) were analyzed by individual ANOVA with main effects of gelding and minute of sampling; the interaction served as the error term for the main effects. Post-treatment means were compared to the pretreatment mean by the least-significant difference test (pdiff option in the least-squares means statement).

2.2. Experiment 2. Exercise Responses

The same nine geldings were used subsequently to test the effects of brief exercise on the same hormones measured in Experiment 1. Exercise has been used as a “stressor” in previous

experiments [12,18,19] and is known to stimulate plasma prolactin and GH concentrations in horses.

On the day of exercise (July 30, 2015), geldings were walked in from pasture at 06:30 and were held in the outdoor chute described in Experiment 1. Beginning at 07:00, one at a time, geldings were 1) bled via jugular venipuncture (pre-exercise sample), 2) walked approximately 30 m to a round pen, and then 3) exercised for 2 min at a trot, with occasional cantering, on a lunge line. They were encouraged to run via voice signals and a lunge whip, although no physical contact with the whip was used. Once the 2 min had elapsed, the gelding was walked back to a three-sided shed and was tethered to a post for blood sampling. Samples of jugular blood were collected at 5, 10, 20, 30, and 45 min relative to the onset of exercise. Sampling and sample handling were the same as described in Experiment 1. All geldings were done with exercising and blood sampling by 10:00.

Plasma samples were measured for the same hormones as described for Experiment 1. Data for each hormone were analyzed in ANOVA as described for the non-prolactin hormones in Experiment 1 (horse and minutes as main effects).

2.3. Prolactin Response to Sulpiride

All geldings were subjected to a sulpiride challenge on August 4, 2015, so that the magnitude of the prolactin response could be compared directly to the responses to substance P and exercise. All geldings were walked in from pasture that morning and were held in the outdoor chute. After a 0-min blood sample was drawn by jugular venipuncture, each gelding was administered sulpiride (+/- racemic mixture) intravenously at 0.1 mg/kg of body weight. The sulpiride was prepared in saline at 10 mg/mL such that a 500 kg gelding received 5 mL. Post-

injection blood samples were collected at 5, 10, 20, 30, and 45 min after injection. Blood samples were handled and assayed for prolactin as described in Experiment 1. Data were analyzed by ANOVA as described for the non-prolactin hormones in Experiment 1.

3. Results

3.1. Experiment 1

3.1.1. Physiologic Responses to Substance P

Within 20 to 30 s after the intravenous administration of 500 µg of substance P, the first treated gelding began displaying the following external responses: facial contraction and flehmen response, rhinorrhea, salivation, sweating, stretching of the hind legs, looking back at flank, defecation. These responses were present in all geldings receiving the 500 and 250 µg doses. In general, most of the responses subsided within 5 to 10 min. Heart rate and respiration rate were assessed in some geldings, and were elevated in the first few minutes after substance P injection, but subsided quickly. After the bulk of responses subsided, most geldings displayed a general depression, with head down and minimal movement. This subsided as well by 15 min after injection. Geldings receiving the lower doses (125 and 62.5 µg) of substance P displayed sweating and rhinorrhea in most cases, with fewer displays of flehmen and salivation. In addition, any responses tended to subside faster in geldings administered the lower doses. When geldings were administered saline only, they displayed none of these responses.

3.1.2. Blood and Hormonal Responses

When plasma samples were centrifuged to harvest plasma, there was an obvious reduction in the volume of plasma harvested after decanting samples collected at 10 and 20-min

post-treatment (relative to pretreatment samples) for geldings that had received the higher doses of substance P. This reduction became gradually less obvious in the later time periods and was not apparent at all in samples from saline-treated geldings or those receiving the 62.5 µg dose of substance P.

The mean plasma prolactin responses to saline and the four doses of substance P are presented in Fig 1. Figure 1A summarizes the results of the analysis of the natural logs of prolactin concentrations; the means in Figure 1C are from the exponential transformation of the log means in Figure 1A. Analysis of the natural logs indicated a dose-by-day interaction ($P = 0.0062$) for prolactin concentrations, which was mainly due to the higher doses of substance P. Further analysis of the net changes in prolactin concentrations (Fig 1B and 1D) separately for the lowest dose and again for the three highest doses revealed that all doses of substance P increased ($P < 0.01$) prolactin concentrations relative to pretreatment means (and versus controls for the 62 µg dose). However, there was no difference ($P > 0.1$) among the prolactin responses for the three highest doses (125, 250, and 500 µg).

Plasma concentrations of all six adenohipophyseal hormones in response to the 250 µg dose of substance P are presented in Fig 2. Concentrations of GH ($P = 0.05$), ACTH ($P < 0.0001$) and prolactin ($P = 0.079$) all increased within 10 min after substance P administration. Concentrations of LH ($P = 0.14$), FSH ($P = 0.40$), and TSH ($P = 0.82$) were not altered by substance P administration.

3.2. Experiment 2. Exercise Responses

Plasma concentrations of all six adenohipophyseal hormones in response to a 2-min exercise bout are presented in Fig 3. Concentrations of GH ($P = 0.0035$), ACTH ($P = 0.0002$)

prolactin ($P < 0.0001$), and LH ($P = 0.055$) all increased within 10 min after onset of exercise; concentrations of GH and prolactin stayed elevated up to 45 min after onset of exercise. Concentrations of FSH ($P = 0.71$) and TSH ($P = 0.18$) were unaffected by exercise.

3.3. Experiment 3. Prolactin Response to Sulpiride

Plasma prolactin concentrations in response to a fixed dose of sulpiride (0.1 mg/kg of body weight) are presented in Fig 4. Prolactin concentrations increased ($P < 0.0001$) within 5 min after sulpiride injection and remained elevated through 45 min after injection.

3.4. Relationships among Hormonal Responses

There was no significant correlation among the net prolactin responses (highest concentration minus the 0 min concentration) following substance P administration (at the 250 μ g dose), exercise, and sulpiride administration (data not shown). Removal of data for two geldings that had the greatest prolactin response to 250 μ g substance P (77 and 367 ng/mL compared to 11.4 ± 1.9 for the remaining 7 geldings) only altered the relationship between the prolactin response after substance P and after sulpiride ($r = 0.641$, $P = 0.06$).

4. Discussion

Thompson and Oberhaus [4] pointed out in their 2015 review of prolactin research in horses that a special relationship exists between estradiol stimulation of the adenohypophysis and the subsequent secretion of prolactin. Specifically, estradiol pretreatment of mares or geldings greatly enhances the prolactin response to either sulpiride or domperidone (i.e., dopaminergic antagonism), whereas other stimuli for prolactin release (e.g., TRH injection, exercise, PGF2 α

injection) do not synergize in the same way with estradiol [19]. This dichotomy may be due to different pathways via which prolactin is secreted: via a drop in dopaminergic input to the lactotropes from the hypothalamic-hypophyseal portal blood supply versus a possible direct neural innervation. Given that stressors typically cause a mass discharge of the sympathetic nervous system (at least to some degree, depending upon the intensity of the stressor[20]), a rapid neural input would be a logical alternative mode of regulation for prolactin, as well as GH and ACTH, release from the adenohypophysis.

Administration of substance P intravenously certainly did result in many of the responses that would normally occur with acute stress. In addition to the prolactin, GH, and ACTH responses, sweating and increased heart rate (indicative of epinephrine release) were observed almost immediately. Again, this would be consistent with a mass discharge effect that typically would involve rapid nervous stimulation of the adrenal medulla [20]. In addition, the sweating, diarrhea, and signs of colic displayed by the geldings receiving the higher three doses of substance P may indicate a release of PGF2 α , given that these side effects are often seen when PGF2 α is administered to mares for luteolysis [21,22].

Although salivation is usually associated with enhanced parasympathetic activity, the parotid and submaxillary glands of the rat have been shown [23] to respond to substance P administration with a cholinergic-like response (stimulation of secretion). Others [24] have reported specific binding of iodinated substance P analogs to rat submaxillary gland homogenates, indicating receptors specific for substance P. Thus the excessive salivation observed in the geldings administered the higher doses was likely due to a direct stimulatory effect of the injected substance P on the salivary glands. Regarding the rhinorrhea observed in those geldings receiving the higher doses of substance P, substance P receptors have been

reported to be located around submucosal glands in guinea pig nasal mucosa [25], and may be involved with histamine-induced nasal discharge. Studies with humans have indicated a substance P involvement with nasal secretions [26,27]. Direct study in horses will be needed to clarify the role of substance P in nasal fluid secretory rates.

The general model for mass discharge of the sympathetic nervous system in response to a significant peripheral damage or painful stimulus (the proverbial tiger on the horse's back) includes transmission of painful stimuli up nociceptor afferents (myelinated A- δ and non-myelinated C fibers) into the dorsal horn of the spinal cord where they synapse with ascending spinothalamic tracts that relay the stimulus up the spinal cord [28]. Substance P and glutamate are the two neurotransmitters involved with those synapses [28,29]. Assuming substance P administered via the jugular vein reaches these synapses in the dorsal horn, the effects of exogenous substance P could be primarily occurring at this level, with stimulation of the postsynaptic fibers and a transmission of a "pain" signal up the spinal cord to the brain. Such an interaction at this level would be perceived as pain but in the absence of any damage to peripheral tissues. If this were the case in Experiment 1, it might be expected that the geldings would react (behave) in a manner indicative to exposure to a painful stimulus. Given that they did not act startled or "excited" as they would from a direct noxious stimulus (i.e., there was no flight-or-fight behavior), it is likely that they were either not experiencing a general pain perception, or alternatively, such a perception was not great enough at the doses applied to cause a fearful reaction. Infusion of a substance P agonist in men [30] at a rate of up to 8 pmol/kg/min for 20 min did not affect pulse rate or blood pressure, but did stimulate lacrimation and flushing (reddening) of the face; no detrimental side effects were reported. In another report of substance

P infusion in men [31], increases in plasma ACTH and GH concentrations were observed at a lower dose (1.5 pmol/kg/min).

The decrease in harvested plasma volume observed in the jugular blood samples after the higher doses of substance P was similar to that observed after acute exercise [32]. The decrease would be indicative of an increase in packed red cell volume, which is known to occur after exercise due to fluid flux into the nonvascular spaces due to the hydrostatic pressure increase created by peripheral vasoconstriction [32]. Again, this is likely a sign of epinephrine (and norepinephrine) secretion from the adrenal medullae in response to substance P administration. These responses (sweating, colic symptoms, shifts in packed red cell volume) are also very similar to those observed by Sticker et al [16] for mares infused intravenously with neutralized aspartic acid. Those effects were reported to peak about 5 to 10 min after the start of infusion and gradually faded over the next 10 min. Those results are consistent with the model of pain transmission [28,29] that includes glutamate secretion at the terminal buttons of the primary afferents in the dorsal horn of the spinal cord (in addition to substance P). Receptors for both glutamate and substance P are located in various regions of the brain as well, and are known to be involved in the process of pain reception and the physiologic responses to it [9-11].

Specific saturable high affinity receptors for substance P have been reported for adenohypophyseal cells of the human [33] and rat [34-36]. Substance P itself has been localized in neurons of the median eminence, neurohypophysis and adenohypophysis of the rat [36]. Moreover, it has been reported that substance P stimulates prolactin secretion directly in cultured-then-perifused rat pituitary cells [37]. Thus, the possible involvement of substance P as the mediator of prolactin secretion in response to exercise in horses is a viable alternative to the “reduction in dopaminergic input” hypothesis that is often described when discussing the

regulation of prolactin secretion [4]. Confirmation (or refutation) of a direct effect of substance P on prolactin secretion from equine lactotrope in vitro, similar to the experiments of Henriksen et al [37], should be a high priority for future research in this area.

The response of adenohypophyseal hormones to acute exercise in Experiment 2 was very similar 1) to that reported previously [4], and 2) to the responses to substance P administration. Prolactin, GH, and ACTH all increased either immediately or soon after exercise was initiated. Although separation in time is a confounding factor (exercise was performed after all substance P data had been collected), the magnitude of response of ACTH and prolactin concentrations to exercise was much less than after injection of 250 µg of substance P; GH responses were similar to the two stimuli. The minor increase in LH concentrations after exercise was not evident after substance P administration. The 2-min exercise bout, although known from previous studies to cause a rise in GH and prolactin, did not result in profuse sweating in the geldings in Experiment 2, and thus was not that great a stressor in general. This may account for the differences in magnitude of the ACTH and prolactin responses between exercise and substance P administration. Regardless, the positive responses in GH, ACTH, and prolactin to the two different stimuli is consistent with a potential model in which substance P is an intermediate in the exercise responses.

Experiment 3, in which the same geldings were administered sulpiride, was performed to determine whether correlations existed among prolactin responses to the three distinct prolactin secretagogues. Again, although separated in time, it's fair to assume that high versus low secretors of prolactin in one case would display the same characteristic in the other 2 cases. This assumption is somewhat supported by the fact that repeated injections of sulpiride, every-other-day in mares, produced relatively consistent prolactin responses over a 4-week period [38]. As

mentioned earlier, there were two geldings in Experiment 1 that had far greater prolactin responses to 250 µg of substance P than the other seven geldings (by a factor of 7 and 36). Surprisingly, their prolactin responses to exercise and sulpiride were not extraordinary. In all nine geldings, the prolactin response to 250 µg of substance P was similar to or greater than the prolactin response to exercise. Except for the two geldings mentioned, all the other geldings' prolactin responses to sulpiride were much greater than their responses to substance P. Accordingly, there were no correlations among the three responses unless the two high prolactin-secreting geldings (after substance P) were ignored, in which case there was a moderate correlation between the sulpiride responses and the substance P responses.

In conclusion, the data generated in these experiments are consistent with a possible role of substance P in the prolactin response to stressful stimuli. From the literature, potential locations of interaction include the substance P synapses in the spinal cord, various areas of the brain, as well as the adenohypophysis itself.

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References

- [1] Rabb MH, Thompson Jr DL, Barry BE, Colborn DR, Garza Jr F, Hehnke KE. Effects of sexual stimulation, with and without ejaculation, on serum concentrations of LH, FSH, testosterone, cortisol and prolactin in stallions. *J Anim Sci* 1989;67:2724–9.
- [2] Colborn DR, Thompson Jr DL, Roth TL, Capehart JS, White KL. Responses of cortisol and prolactin to sexual excitement and stress in stallions and geldings. *J Anim Sci* 1991;69:2556–62.
- [3] Thompson Jr DL, DePew CL, Ortiz A, Sticker LS, Rahmanian MS. Growth hormone and prolactin concentrations in plasma of horses: sex differences and the effects of acute exercise and administration of growth hormone releasing hormone. *J Anim Sci* 1994;72:2911–8.
- [4] Thompson Jr DL, Oberhaus EL. Prolactin in the horse: Historical perspective, actions and reactions, and its role in reproduction. *J Equine Vet Sci* 2015;35:343–53.
- [5] Zhang P, Liu L, Xie CJ, Wang KH, Gao LZ, Ju G. Excitatory and inhibitory effects of prolactin release activated by nerve stimulation in rat anterior pituitary. *Reprod Biol Endocrinol* 2009;7:154 (doi: 10.1186/1477-7827-7-154).
- [6] Ju G, Liu S. Relationship of substance P immunoreactive nerve fibers with somatotropes of the anterior pituitary in the monkey. *J Neuroendocrinol* 1989;1:397-400.
- [7] Ju G, Liu SJ. Substance P-like immunoreactive nerve fibers in the pars distalis of the anterior pituitary in the dog. *Cell Tissue Res* 1990;261:323-31.
- [8] Liu S. Peptidergic innervation in pars distalis of the human anterior pituitary. *Brain Res*. 2004;1008:61-8.
- [9] Mistrova, E, Kruzliak P, Chottova Dvorakova M. Role of substance P in the cardiovascular system, *Neuropeptides* 2015;<http://dx.doi.org/10.1016/j.npep.2015.12.005>

365 [10] Onaga T. Tachykinin: recent developments and novel roles in health and disease. *Biomol*
366 *Concepts* 2014;5:225-43.

367 [11] Howard MR, Haddley K, Thippeswamy T, Vasiliou S, Quinn P. Substance P and the
368 tachykinins. In: Lajtha A, Lim R, editors. *Handbook of neurochemistry and molecular*
369 *neurobiology: Neuroactive proteins and peptides*. 3rd Ed. New York: Springer; 2006, p. 427-62.

370 [12] Colborn DR, Thompson Jr DL, Roth TL, Capehart JS, White KL. Responses of cortisol and
371 prolactin to sexual excitement and stress in stallions and geldings. *J Anim Sci* 1991;69:2556–62.

372 [13] Thompson DL Jr, Rahmanian MS, DePew CL, Burleigh DW, DeSouza CJ, Colborn DR.
373 Growth hormone in mares and stallions: Pulsatile secretion, response to growth hormone-
374 releasing hormone and effects of exercise, sexual stimulation, and pharmacological agents. *J*
375 *Anim Sci* 1992;70:1201-7.

376 [14] Thompson DL Jr, Godke RA, Squires EL. Testosterone effects on mares during
377 synchronization with altrenogest: FSH, LH, estrous duration and pregnancy rate. *J Anim Sci*
378 1983;56:678-86.

379 [15] Thompson DL Jr, Reville SI, Walker MP, Derrick DJ, Papkoff H. Testosterone
380 administration to mares during estrus: Duration of estrus and diestrus and concentrations of LH
381 and FSH in plasma. *J Anim Sci* 1983;56:911-8.

382 [16] Sticker LS, Thompson Jr DL, Gentry LR. Pituitary hormone and insulin responses to
383 infusion of amino acids and Nmethyl-D,L-aspartate in horses. *J. Anim. Sci.* 2001;79:735–44.

384 [17] Steel RGD, Torrie JH, Dickey DA. *Principles and Procedures of Statistics: A Biometrical*
385 *Approach* (3rd ed). McGraw-Hill Book Co, 1997.

386 [18] Thompson Jr DL, DePew CL, Ortiz A, Sticker LS, Rahmanian MS. Growth hormone and
387 prolactin concentrations in plasma of horses: Sex differences and the effects of acute exercise
388 and administration of growth hormone releasing hormone. *J Anim Sci* 1994;72:2911-8.

389 [19] Thompson Jr DL, Clavier SC, Mitcham PB, Earl LR. Estradiol effects on secretagogue
390 induced prolactin release: disparity in responses to sulpiride, exercise, epinephrine,
391 prostaglandin-F2alpha, and thyrotropin releasing hormone. *J Equine Vet Sci* 2013;33:607–14.

392 [20] Guyton AC, Hall JE. *Textbook of Medical Physiology*. 12th edition. Philadelphia:
393 W.B. Saunders, 2011. p. 729-742.

394 [21] Klem ME Jr, Kreider JL, Harms PG, Potter GD, Kraemer DC, Godke RA. Induction of
395 parturition in the mare with prostaglandin F2-alpha. *Prostaglandins* 1982;24:89-96.

396 [22] Irvine CH, McKeough VL, Turner JE, Alexander SL, Taylor TB. Effectiveness of a two-
397 dose regimen of prostaglandin administration in inducing luteolysis without adverse side effects
398 in mares. *Equine Vet J* 2002;34:191-4.

399 [23] Yu JH, Burns SM, Schneyer CA. Salivary secretion induced by substance P. *Proc Soc Exp*
400 *Biol Med* 1983;173:467-70.

401 [24] Bahouth SW, Stewart JM, Musacchio JM. Specific binding of a 125I-labeled substance P
402 analog to rat submaxillary gland. *J Pharmacol Exp Ther* 1984;230:116-23.

403 [25] Shibano A1, Kawai Y, Senba E. Histamine-induced internalization of substance P receptors
404 in myoepithelial cells of the guinea pig nasal glands. *Peptides* 1998;19:1365-71.

405 [26] Mullol J, Rieves RD, Baraniuk JN, Lundgren JD, Mérida M, Hausfeld JH, Shelhamer JH,
406 Kaliner MA. The effects of neuropeptides on mucous glycoprotein secretion from human nasal
407 mucosa in vitro. *Neuropeptides* 1992;21:231-8.

408 [27] Fang SY, Shen CL. Neuropeptidergic innervation of human nasal mucosa in various
409 pathological conditions. *Proc Natl Sci Counc Repub China B*. 1997;21:8-12.

410 [28] Khasabov SG, Rogers SD, Ghilardi JR, Peters CM, Mantyh PW, Simone DA. Spinal
411 neurons that possess the substance P receptor are required for the development of central
412 sensitization. *J Neurosci* 2002;22:9086-98.

413 [29] Willis WD. Role of neurotransmitters in sensitization of pain responses. *Ann NY Acad Sci*
414 2001;933:142-56.

415 [30] Schaffalitzky De Muckadell OB, Aggestrup S, Stentoft P. Flushing and plasma substance P
416 concentration during infusion of synthetic substance P in normal man. *Scand J Gastroenterol*
417 1986;21:498-502.

418 [31] Volpi R, Caffarra P, Scaglioni A, Saginario A, Maestri D, Vourna S, Vescovi PP, Chiodera
419 P, Coiro V. Lack of ACTH/cortisol and GH responses to intravenously-infused substance P in
420 Parkinson's disease. *J Neural Transm Park Dis Dement Sect* 1993;6:99-107.

421 [32] McKeever KH, Hinchcliff KW, Reed SM, Robertson JT. Role of decreased plasma volume
422 in hematocrit alterations during incremental treadmill exercise in horses. *Am J Physiol*
423 1993;265:R404-8.

424 [33] Wormald PJ, Millar RP, Kerdelhué B. Substance P receptors in human pituitary: a potential
425 inhibitor of luteinizing hormone secretion. *J Clin Endocrinol Metab* 1989;69:612-5.

426 [34] Kerdelhué B, Tartar A, Lenoir V, El Abed A, Hublau P, Millar RP. Binding studies of
427 substance P anterior pituitary binding sites: changes in substance P binding sites during the rat
428 estrous cycle. *Regulatory Peptides* 1985;10:133-43.

429 [35] Larsen PJ, Mikkelsen JD, Mau S, Saermark T. Binding and internalization of a iodinated
430 substance P analog by cultured anterior pituitary cells. *Mol Cell Endocrinol*. 1989;65:91-101.

431 [36] Mikkelsen JD, Larsen PJ, Møller M, Vilhardt H, Saermark T. Substance P in the median
432 eminence and pituitary of the rat: demonstration of immunoreactive fibers and specific binding
433 sites. *Neuroendocrinology* 1989;50:100-8.

434 [37] Henriksen JS, Saermark T, Vilhardt H, Mau SE. Tachykinins induce secretion of prolactin
435 from perfused rat anterior pituitary cells by interactions with two different binding sites. *J*
436 *Recept Signal Transduct Res* 1995;15:529-41.

437 [38] Hebert RC, Thompson Jr DL, Mitcham PB, Lestelle JD. Repeatability of prolactin
438 responses to a small dose of sulpiride in estrogen primed geldings in spring and in mares during
439 the estrous cycle in summer. *J Equine Vet Sci* 2013;33:683–9.

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Figure legends:

Fig 1. Plasma prolactin concentrations in geldings immediately before (time 0) and after intravenous injection of 0, 62, 125, 250, or 500 µg of substance P (SP). Data were transformed for analysis due to heterogeneity of variances to either natural logs of the original data, or to net change in concentrations above the time 0 concentrations. Geometric means were derived as the exponents of the mean natural logs. The net changes were analyzed in two separate analyses due to the large variations in responses to the three highest doses of substance P. Prolactin increased ($P < 0.05$) in response to all doses except the 0 µg (saline control). There was no difference ($P > 0.1$) among the responses of the three highest doses. Asterisks indicate differences ($P < 0.05$) between means for treated geldings (all doses) and those of controls. Pooled standard errors of the arithmetic means were 1.4 ng/mL for the controls versus the 62 µg dose, and 18.8 ng/mL for the three highest doses.

Fig 2. Plasma concentrations of growth hormone (GH), adrenocorticotrophic hormone (ACTH), prolactin, luteinizing hormone (LH), follicle stimulating hormone (FSH), and thyroid stimulating hormone (TSH) in geldings immediately before (time 0) and after intravenous injection of 250 µg of substance P (SP) in Experiment 1. There was an effect of time for GH ($P = 0.05$), ACTH ($P < 0.0001$), and prolactin ($P = 0.08$) but not ($P > 0.1$) LH, FSH, or TSH concentrations. Pooled standard errors of the means were: GH, 5.7 ng/mL; ACTH, 24.5 pg/mL; prolactin, 22.0 ng/mL; LH 0.19 ng/mL; FSH, 0.92 ng/mL; and TSH, 0.09 ng/mL. * $P < 0.05$, + $P < 0.1$, means differ from mean concentrations at time 0.

464

465 Fig 3. Plasma concentrations of growth hormone (GH), adrenocorticotrophic hormone (ACTH),
466 prolactin, luteinizing hormone (LH), follicle stimulating hormone (FSH), and thyroid stimulating
467 hormone (TSH) in geldings immediately before (time 0) and after 2 min of acute exercise in
468 Experiment 2. There was an effect of time for GH ($P = 0.0035$), ACTH ($P = 0.0002$), prolactin (P
469 < 0.0001), and LH ($P = 0.055$) but not ($P > 0.1$) FSH or TSH concentrations. Pooled standard
470 errors of the means were: GH, 4.4 ng/mL; ACTH, 2.5 pg/mL; prolactin, 0.8 ng/mL; LH 0.16
471 ng/mL; FSH, 0.67 ng/mL; and TSH, 0.08 ng/mL. * $P < 0.05$, + $P < 0.1$, means differ from mean
472 concentrations at time 0.

473

474 Fig 4. Plasma concentrations of prolactin in geldings in response to an intravenous injection of
475 sulpiride (racemic mixture) at 0.1 mg/kg of body weight in Experiment 3. There was an effect of
476 time ($P < 0.0001$) in the analysis. The pooled standard of the means was 3.9 ng/mL. * $P < 0.05$,
477 means differ from mean concentrations at time 0.

478

FIGURES

Figure 1

Figure 2

Figure 3

Figure 4