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The Effects of Tryptophan and Manipulations of Serotonergic Receptors on Tonic Immobility in Chickens[1]

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WALLNAU, L B., G D BORDASH AND P. CORSO, JR. *The effects of tryptophan and manipulations of serotonergic receptors on tonic immobility in chickens* PHARMAC BIOCHEM. BEHAV. 14(4) 463–468, 1981 —The effects of serotonergic manipulations on tonic immobility (TI) were examined. Systemic injections of tryptophan enhanced TI duration This effect was reversed by quipazine, a 5-HT receptor agonist, and p-chloroamphetamine, a 5-HT releaser Separately, these drugs caused marked reductions in TI duration. Fenfluramine, which promotes 5-HT release, also reduced TI duration The quipazine attenuation of TI was prevented by pretreatment with the 5-HT receptor blocker cinanserin The results are discussed in terms of 5-HT receptor mechanisms and the raphe model of tonic immobility.

Tonic immobility Serotonergic system Raphe model Tryptophan Quipazine p-Chloroamphetamine Fenfluramine Cinanserin Chickens

BRIEF physical restraint will often elicit a motionless catatonic-like reaction in many animals. This response, known as tonic immobility (TI), may last from a few seconds to as long as several hours after the initial restraint. It is characterized by waxy flexibility, tremors of the extremities, rigidity, eye closure, mydriasis, decreased vocalization and physiological responses such as changes in heart and respiration rates, core temperature, and EEG patterns [16]. The reaction terminates abruptly and often is followed by apparent defensive and escape responses [16]. Domestic fowl are typically utilized in the study of TI because of the ease with which the response can be elicited and quantified.

A serotonergic mechanism appears to participate in TI and a serotonergic-raphe model has been proposed for predicting drug effects on tonic immobility [34]. Specifically, drugs that are known to inhibit the firing rate of central serotonergic neurons of the midbrain raphe nuclei in mammals (e.g., LSD, tryptophan, pargyline) potentiate TI duration in domestic fowl [1–5, 10, 15, 17, 23]. The mechanism was presumed to involve the triggering of a negative neuronal feedback mechanism due to excess synaptic concentrations of serotonin (5-HT) that would result from certain drug manipulations (e.g., reuptake inhibition, monoamine oxidase inhibition, promotion of 5-HT release, precursor loading). It was also noted in the original model that the putative relationship between raphe firing and TI duration is bidirectional. That is, drugs which enhance raphe activity (e.g., amphetamine) produce a decrement in tonic immobility [9,13].

Recent findings [10, 20, 33] have raised questions regarding the original formulation of the raphe model, and shifted the emphasis to the postsynaptic consequences of 5-HT manipulations For example, the monoamine oxidase (MAO) inhibitor pargyline or the 5-HT precursor tryptophan will inhibit raphe firing rate [1, 3, 15] and increase TI duration [10, 17, 23], but combined administration of these drugs produce a marked reduction in response duration [10]. These findings seem to be paradoxical and cannot be readily incorporated into the original raphe model. However these results might be better understood by considering the regulatory influences on raphe activity and the postsynaptic consequences of drug manipulations of the serotonergic system [10].

Aghajanian and Wang [8] have re-examined the nature of the regulatory mechanisms of serotonergic neurons and have suggested that raphe activity is self-regulated rather than modulated by negative neuronal feedback. The autoregulatory mechanism could produce changes in raphe firing via collaterals intrinsic to the raphe system and by autoreceptors on the presynaptic membrane that are sensitive to the presence of 5-HT [8]. It is interesting that transection studies have cast doubt on the existence of negative neuronal feedback from postsynaptic regions as an important source of regulation [27]. On the other hand, the existence of collaterals intrinsic to the raphe system is supported by retrograde tracing techniques [7,25] and recurrent inhibition in the midbrain serotonergic system [35]. Furthermore, the effects of microiontophoretically applied drugs (e.g., LSD, 5-HT) on single raphe units have supported the presence of presynaptic autoreceptors [4,5].

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In light of these findings, a functional distinction might be made between pre- and postsynaptic effects of drugs on 5-HT mechanisms. Aghajanian and Wang [8] have pointed to a possible asymmetry between drug effects on raphe firing rate and the postsynaptic consequences of these manipulations. That is, drugs which facilitate 5-HT availability and synaptic functioning should inhibit raphe activity (presumably by a presynaptic mechanism), and place postsynaptic neurons in other brain areas receiving 5-HT input under tonic inhibition [8]. On the other hand, drugs which produce a direct inhibition of raphe activity should impair 5-HT transmission and have the opposite effect of releasing postsynaptic neurons from tonic inhibition [8]. Thus, while both instances involve an inhibition of raphe firing, the postsynaptic effects are different. With this asymmetry in mind, it has been suggested [10] that singular administration of either pargyline or tryptophan may exert its influence by inhibiting raphe firing directly, resulting in a decrease in 5-HT input to postsynaptic regions and an increase in TI duration. It is significant in this regard that microiontophoretically applied tryptophan inhibits raphe firing [15]. Furthermore, the tryptophan effect on raphe units is probably not due to mechanisms involving raphe terminals. Specifically, p-chlorophenylalanine (PCPA), which is more effective in blocking 5-HT synthesis in raphe terminals than perikarya [6], fails to prevent the inhibitory influence of systemic injections of tryptophan on raphe firing [1,15]. The paradoxical reduction of TI following the combined treatment of pargyline and tryptophan might be due to an increase in synaptic transmission of 5-HT that would not otherwise be present with singular administration of these agents [10]. In this regard, it has been suggested [18] that tryptophan allows for the synthesis of 5-HT in excess of the capacity for its intraneuronal storage. When the catabolism of this excess 5-HT is prevented by MAO inhibitors such as pargyline, a "spill-over" of 5-HT onto postsynaptic receptor sites may result [18]. Thus while direct inhibition of raphe activity (e.g., by tryptophan) would reduce 5-HT release and increase TI, manipulations which facilitate the synaptic transmission of 5-HT (e.g., a "spill-over" of 5-HT due to precursor loading combined with MAO inhibition) abbreviate TI duration.

The importance of postsynaptic effects of serotonin is also supported by the finding that quipazine, a putative 5-HT receptor agonist [21,29], reduces TI duration [33]. Consistent with this finding, d-amphetamine and peripherally administered 5-HT, which augment raphe firing rate [13,26] and thus presumably 5-HT release and postsynaptic function, also produce shortened durations of immobility [9,23]. In addition, inhibition of 5-HT synthesis by PCPA prevents the amphetamine reduction in tonic immobility [9]. Underscoring the possible importance of the postsynaptic consequences of serotonergic manipulations, LSD directly and preferentially inhibits raphe activity [4,19], results in disinhibition of postsynaptic areas receiving heavy 5-HT input [19], and potentiates the duration of tonic immobility [23]. It is interesting that a dose-dependent biphasic effect of 5-HT on TI has been reported [20], with low doses of 5-HT prolonging and high doses abbreviating response duration. Although it is difficult to interpret this effect in terms of the original raphe model, it may reflect a difference between pre- and postsynaptic mechanisms. That is, perhaps low doses of 5-HT preferentially inhibit 5-HT neurons directly (e.g., [4,5]), thus reducing postsynaptic serotonergic function. On the other hand, large doses of 5-HT may enhance postsynaptic function due to a "spill-over" of excess 5-HT onto postsynaptic sites (e.g., [18]), or by enhanced raphe activity (e.g., [26]).

Thus, in contrast to the original formulation of the serotonergic-raphe model of TI [34], it appears that a drug effect on raphe activity does not predict TI duration as well as the postsynaptic consequences of that drug manipulation. In an attempt to further clarify the role of the serotonergic system for TI, the present study examines the effects of manipulations of 5-HT receptors on tonic immobility.

EXPERIMENT 1

Tryptophan, when systemically or iontophoretically injected, produces inhibition of serotonergic neurons of the midbrain raphe nuclei [1,15]. Systemic injections of the 5-HT precursor enhance durations of tonic immobility in chickens [10,17]. If the increment in TI duration reflects a decrease in 5-HT input to postsynaptic regions following the direct inhibition of raphe activity, then stimulation of postsynaptic serotonergic receptors by quipazine [21,29] should prevent the tryptophan potentiation of TI duration.

METHOD

Animals Forty straight-run Production Red chickens were acquired from a commercial hatchery (Welp, Inc.), at one day of age. The animals were group housed in brooders (Brower Mfg. Co., Model 6401) and had continuous access to food and water. Artificial illumination was provided in the colony room from 8 a.m. to 10 p.m. daily. Human exposure was limited to routine daily care because familiarization and habituation to humans can reduce TI duration [28].

Apparatus. The duration of TI was timed automatically by electronic programming equipment. Photoelectric sensors (BRS/LVE PH-901/221-10) detected the termination of the response. Tonic immobility was elicited by restraining the animal on its side on a wood platform. Placing the back of the animal against a positioning block located on the platform allowed accurate and reliable recording of the righting response. The platform (HWD=10×42×30 cm) was housed in a modified sound-attenuated test chamber (HWD=58×105 ×41 cm). A photoelectric cell was situated in the platform so that it would be below the immobile animal, and the light source was on the ceiling of the chamber Provided an animal displayed TI after restraint, a switch was activated by the experimenter which started an electronic clock and a sound-attenuated door was closed. Upon righting to its feet, the animal would allow contact to be restored between the photoelectric cell and the light source, which in turn would stop the clock. A locking relay prevented additonal time recording if the animal broke the beam by movements following the righting response. Thus, only the duration of TI, from release until the righting response had occurred, was recorded by the apparatus. Small windows on the test chambers allowed observation, and were situated so that the observer would be out of view of the immobilized animal.

Procedure. At 24 days of age, animals were assigned to one of four groups that received two injections consisting of tryptophan and quipazine, tryptophan and vehicle, vehicle and quipazine, and two vehicle injections. For the first injection, animals received either 400 mg/kg IP of L-tryptophan methyl ester HCL (U. S. Biochemical) or an equivalent volume of distilled water. The birds were placed in cardboard holding boxes for 15 min and were subsequently treated with 25 mg/kg IP of quipazine maleate (Miles

TABLE 1

THE EFFECTS OF TRYPTOPHAN (400 mg/kg) AND QUIPAZINE (25 mg/kg) ON TONIC IMMOBILITY

		Vehicle-Vehicle	Tryptophan-Vehicle	Vehicle-Quipazine	Tryptophan-Quipazine
Duration (sec)	Mean	571 20	2274.50	25 50	20.80
	Standard Error	112.65	659 52	17.98	10 17
Number of Inductions	Mean	1 30	1 20	3.30	3.30
	Standard Error	0.21	0.20	0 52	0 63

TABLE 2

THE EFFECTS OF CINANSERIN (50 mg/kg) AND QUIPAZINE (25 mg/kg) ON TONIC IMMOBILITY

		Vehicle-Vehicle	Cinanserin-Vehicle	Vehicle-Quipazine	Cinanserin-Quipazine
Duration (sec)	Mean	900 33	1389 43	1.33	1703.67
	Standard Error	507 86	457.74	1 34	1041 74
Number of Inductions	Mean	1 33	1.14	5 0	2.0
	Standard Error	0.33	0 16	0	0 63

Laboratories) or the distilled water vehicle. All injection volumes were 3.33 ml/kg. Each animal was returned to the holding boxes for an additional 15 min and then individually tested for TI by applying 15 sec of restraint on its side in the apparatus described above. If the initial restraint did not elicit immobility, the animal was returned to the holding box for 60 sec and restraint was repeated. This procedure was repeated until TI was displayed or five unsuccessful induction attempts had occurred. Animals that did not display immobility after five elicitation attempts received a duration score of zero sec. Individuals testing for TI were kept uninformed of the group designations of the animals. Testing was performed between 10 a.m. and 4 p.m. and each group was equally distributed across the time of day that testing was conducted.

RESULTS

Table 1 depicts the results of the first experiment. A square root transformation was performed on durations to reduce skew and alleviate heterogeneity of variance. Analysis of variance revealed a main effect for tryptophan, $F(1,36)=6.52$, $p<0.025$, reflecting enhanced durations for this treatment. Quipazine treated animals displayed short durations, as indicated by a main effect, $F(1,36)=59.38$, $p<0.001$. An interaction also occurred between these two treatments, $F(1,36)=6.87$, $p<0.025$. Planned simple effects comparisons were performed to test the specific hypotheses, that tryptophan by itself would increase TI duration relative to controls, and that tryptophan-quipazine animals would resemble those treated with just quipazine. As predicted, the vehicle-vehicle and tryptophan-vehicle treatments differed, $F(1,36)=13.39$, $p<0.001$, indicating that tryptophan by itself enhanced TI duration and replicating previous findings [10,17]. However, there was no reliable difference between the two quipazine treatments ($F<1$). Thus although animals treated with just tryptophan displayed prolonged immobility, the tryptophan-quipazine group displayed short reactions like animals that received only quipazine. An analysis of the number of inductions required to produce TI yielded a main effect for the quipazine conditions $F(1,36)=22.28$, $p<0.001$, indicating that quipazine-injected animals required more elicitation attempts that those not receiving quipazine. The main effect for tryptophan ($F<1$) and the interaction of drug treatments ($F<1$) were not significant.

EXPERIMENT 2

Quipazine, a putative 5-HT receptor agonist [21,29], produces behavioral effects in chickens that closely resemble those following the activation of serotonergic mechanisms in avians [10, 22, 33]. These behaviors include wing abduction, a squatting posture, head arched back with beak gaped, alternate shifting of weight on each foot, and occasional lurching forward [33]. While these observations indirectly support the involvement of 5-HT mechanisms in quipazine-induced behavioral effects in avians, quipazine has been reported to alter the functioning of other neurotransmitter systems [24]. In order to determine if quipazine alters TI by its action on 5-HT receptors, the effect of cinanserin, a 5-HT antagonist [14,30], was examined for quipazine-treated animals.

TABLE 3

THE EFFECTS OF TRYPTOPHAN (400 mg/kg) AND p-CHLOROAMPHETAMINE (8 mg/kg) ON TONIC IMMOBILITY

		Vehicle-Vehicle	Tryptophan-Vehicle	Vehicle-PCA	Tryptophan-PCA
Duration (sec)	Mean	245 70	1869.20	13 80	0.40
	Standard Error	115 01	539.02	9 20	0.39
Number of Inductions	Mean	2.40	2 10	4.50	4.80
	Standard Error	0.58	0.53	0 40	0 20

METHOD

Animals Twenty-five Production Red chickens were acquired and maintained as described in the first experiment.

Procedure. At 24 days of age, animals were assigned to one of four injection conditions, consisting of vehicle-vehicle, cinanserin-vehicle, vehicle-quipazine, or cinanserin-quipazine. Initially birds received 50 mg/kg IP of cinanserin HCL (Squibb) or distilled water. Sixty min later, animals were injected with quipazine maleate (25 mg/kg IP) or the vehicle. All injection volumes were 3.33 ml/kg. Animals were tested for tonic immobility 15 min after the second injection with the same apparatus and procedures outlined in the first experiment. In addition, each bird was observed several minutes prior to testing and the absence or presence of lurching, squatting, and wing abduction was noted. When at least two of these behaviors were observed, animals were scored as displaying the serotonergic syndrome.

RESULTS

Table 2 depicts the findings. Analysis of variance was performed following a square root transformation of durations and revealed a main effect for cinanserin, $F(1,21)=4.50, p<0.05$, indicating that this treatment elevated TI duration. The main effect for quipazine, $F(1,21)=3.27$, and the interaction of cinanserin and quipazine treatments, $F(1,21)=1.45$, failed to achieve statistical significance. Since a main effect for quipazine was not obtained, post-hoc comparisons were made with Duncan's multiple range test. The multiple comparisons indicated that the vehicle-quipazine treatment displayed shorter durations than all other groups ($p<0.05$ for each comparison). There were no differences among the remaining groups. Effects were obtained for the number of inductions required to elicit immobility. Main effects revealed that cinanserin-treated animals required fewer induction attempts, $F(1,21)=20.53, p<0.001$, and quipazine-treated animals required a greater number of elicitation attempts, $F(1,21)=41.40, p<0.001$. There was also an interaction between these treatments, $F(1,21)=15.93$, $p<0.001$. Post-hoc comparisons by Duncan's multiple range test indicated that the vehicle-quipazine group required more induction attempts than the remaining groups ($p<0.01$ for each comparison). There were no other statistically significant comparisons. In terms of the quipazine-induced postural and locomotor reactions, all six vehicle-quipazine and only one of six cinanserin-quipazine animals displayed the syndrome prior to immobility testing ($p<0.05$, two tailed, Fisher's exact probability test). Thus cinanserin not only reversed the effects of quipazine on TI duration and number of elicitation attempts, it also prevented the postural and motor reactions that are typically observed following the activation of 5-HT mechanisms.

EXPERIMENT 3

It has been reported that p-chloroamphetamine (PCA) produces behavioral effects in rats that are characteristic of enhanced 5-HT transmission [32]. While PCA has multiple pharmacological effects, these behavioral effects are apparently the result of a rapid release of 5-HT [32]. Consistent with these findings, pilot investigations indicated that PCA produces behavioral reactions in chickens that are essentially the same as those that follow quipazine treatment. Most pronounced among these behaviors were the squatting posture, gaping of the beak, and wing abduction. In light of these findings, and the quipazine reversal of the tryptophan potentiation of TI, the third experiment examined the effects of PCA and tryptophan on tonic immobility.

METHOD

Animals. Forty Production Red chickens were housed and maintained as previously described.

Procedure. At 23 days of age, animals were assigned to vehicle-vehicle, tryptophan-vehicle, vehicle-PCA, and tryptophan-PCA treatments. The first injection consisted of 400 mg/kg IP of L-tryptophan methyl ester HCL or the vehicle After 15 min animals were treated with 8 mg/kg IP of DL-p-chloroamphetamine HCL (Sigma) or an equivalent volume of distilled water All injection volumes were 4 ml/kg and testing was conducted 15 min following the second injection in the same manner as described in Experiment One.

RESULTS

The findings for TI duration and elicitation attempts are shown in Table 3. Analysis of variance following a square root transformation of durations yielded a main effect for the tryptophan conditions, $F(1,36)=11.06, p<0.01$, indicating an enhancement of TI by tryptophan. A main effect for PCA, $F(1,36)=39.82, p<0.001$, reflected a response decrement for this condition, and an interaction occurred between the two drug treatments $F(1,36)=13.76$, $p<0.001$. Simple effect analyses were performed to clarify the nature of the interaction and to test the specific hypotheses that tryptophan would enhance TI by itself and tryptophan-PCA animals

TABLE 4
THE EFFECT OF FENFLURAMINE ON TONIC IMMOBILITY

		Dose (mg/kg)		
		0	15	25
Duration (sec)	Mean	649.89	439.13	14 75
	Standard Error	231.25	293.62	14 18
Number of Inductions	Mean	1	2 88	3 63
	Standard Error	0	0 61	0 70

would have low durations like those receiving only PCA. As expected, tryptophan-vehicle animals remained immobile longer than the vehicle-vehicle controls, $F(1,36)=24.75$, $p<0.001$, and there was no reliable difference between the vehicle-PCA and tryptophan-PCA groups ($F<1$). Thus, PCA like quipazine reversed the tryptophan enhancement of TI duration.

For number of inductions, a main effect of PCA emerged, $F(1,36)=28.25$, $p<0.001$, indicating that PCA-treated birds required more elicitation attempts before displaying tonic immobility. There was no main effect for tryptophan ($F<1$), nor an interaction of treatments ($F=1.77$) for the induction data

EXPERIMENT 4

Previous studies ([10,33], Experiments 1–3) suggest that manipulations designed to increase 5-HT transmission result in a decrease in TI duration and an increase in the number of induction attempts required to elicit the response. The present experiment examined the effects of fenfluramine, another 5-HT agonist [11,12] on tonic immobility. Fenfluramine, like quipazine and PCA, should produce short durations and increase the difficulty of response elicitation.

METHOD

Animals Twenty-five Production Red chickens were acquired and handled as outlined in the first experiment.

Procedure. At 19 days of age, animals were injected with 15 or 25 mg/kg IP of fenfluramine HCL (A. H Robins). A third group received an equivalent volume (2 ml/kg) of the vehicle. Animals were individually tested for TI 20 min after injection, utilizing the same procedures of the previous experiments. Doses ranging from 0.5 to 5 mg/kg were examined in preliminary work and no effects were observed, thus larger doses were used in the present experiment. In addition, Trulson and Jacobs [32] reported that 15 mg/kg of fenfluramine produced the behavioral syndrome that is characteristic of enhanced 5-HT transmission in all animals tested.

RESULTS

Analysis of variance following a square root transformation indicated that fenfluramine decreased TI duration (Table 4), $F(2,22)=4.59$, $p<0.025$, and did so as a linear function of the dose employed, $F(1,22)=8.80$, $p<0.01$. There was an effect of fenfluramine on number of inductions (Table 4), $F(2,22)=7.39$, $p<0.005$, and the linear component of this effect was also significant, $F(1,22)=14.05$, $p<0.005$. Thus the effect of fenfluramine on TI parallels the findings for other 5-HT agonists [33] in that TI duration is abbreviated and animals are less likely to display TI on early induction attempts. Casual observations indicated that fenfluramine-injected animals, especially at the larger dose, displayed behavioral components that often accompany enhanced 5-HT transmission. As with quipazine [33] and combined administration of pargyline and tryptophan [10], animals displayed the squatting posture and marked wing abduction.

DISCUSSION

The present set of experiments suggest that an increase in postsynaptic function of 5-HT is accompanied by a decrease in TI duration. In addition, animals are less likely to display the immobility response on early elicitation attempts. Quipazine, a 5-HT receptor agonist [21,29], and PCA and fenfluramine, both of which promote 5-HT release [11, 12, 32], abbreviate TI durations and augment the number of inductions required to produce the response.

Since the inhibitory action of tryptophan on raphe cells is direct [15], it might enhance TI due to a decrease in synaptic transmission of 5-HT. This interpretation receives support from the findings that both quipazine and PCA prevented the tryptophan enhancement of immobility duration. Animals treated with tryptophan and quipazine showed short reactions and required more inductions, just like those that received only quipazine. The same pattern emerged in Experiment Three for PCA and tryptophan.

Cinanserin, a 5-HT receptor antagonist [14,30], blocked the quipazine reduction in TI duration and the increase in induction attempts. It also prevented the 5-HT behavioral syndrome which consisted primarily of a squatting posture with the head arched back, wing abduction, and a gaped beak. Thus it appears that the behavioral effects of quipazine depend on 5-HT receptor stimulation. The main effect for cinanserin reflected elevated durations of immobility for animals treated with this drug. This finding is consistent with the observation that 5-HT receptor stimulation attenuates TI, because the postsynaptic effects of cinanserin are opposite to those of quipazine, PCA, and fenfluramine. Cinanserin-treated animals also required fewer induction attempts for TI elicitation.

Fenfluramine and PCA, which cause a rapid release of 5-HT, also produced a behavioral syndrome similar to that observed following treatment with quipazine. Furthermore, although fenfluramine and PCA produce a rapid inhibition (within 30 to 45 sec following IV injection) of raphe activity [31], both drugs decrease TI duration. These findings are inconsistent with the original serotonergic-raphe model, which would have predicted an increase in TI duration following inhibition of raphe firing [34]. However, they are in agreement with the assertions that increased postsynaptic 5-HT function leads to decrements in TI, and impaired 5-HT transmission results in response potentiation.

It is interesting that MAO inhibition combined with tryptophan induces the same behavioral syndrome as 5-HT agonists [10,33] and reduces TI duration [10]. Presumably MAO inhibition following precursor loading results in excess intraneuronal 5-HT which spills onto postsynaptic receptors [18]. The increased synaptic transmission of 5-HT, much like that which results from fenfluramine or PCA, is accompanied by an attenuation of TI duration, even though sepa-

rately MAO inhibition and tryptophan decrease raphe firing [1, 3, 15] and increase TI duration [10, 17, 23]. Thus it appears that the consideration of postsynaptic consequences of 5-HT manipulations, rather than raphe firing *per se*, provides a better predictive framework for drug effects on tonic immobility.

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