ß - Alanine Protects Mice from Memory Deficits Induced By Ageing, Scopolamine, Diazepam and Ethanol

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The present study was undertaken to investigate the effects of ß-alanine (a glycine agonist), on learning and memory in mice. ß-alanine (5, 10, 20 and 40 mg/kg i.p.) was administered for 6 successive days, to young (3 months old) and aged-mice (16 months old). The learning and memory parameters were assessed, using elevated plus-maze and passive-avoidance apparatus. The effect of ß-alanine (20 mg/kg for 6 days) on locomotor function of young and aged mice, was studied using photoactometer, to rule out the increase in locomotor performance of mice. ß-alanine at both the doses (10 and 20 mg/kg), significantly improved learning and memory of young- and aged- mice. ß-alanine also reversed scopolamine (0.4 mg/kg i.p.), ethanol (1.0 g/kg i.p.) and diazepam (1.0 mg/kg i.p.) -induced amnesia in young mice. There was no significant effect of ß-alanine on the locomotor activity of both young and aged mice. The probable underlying mechanism of the memory-enhancing effect of ß-alanine appears to be related to its antioxidant, anti-amyloid and procholinergic activities.

β-alanine is the only naturally occurring beta amino acid. It is an inhibitory amino acid, as well as a glycine agonist[1]. β-alanine crossed the blood brain barrier via the secondary active transport mechanism, that is common to beta-amino acids[2]. Cell damaging conditions such as hypoxia, hypoglycemia, ischemia, and oxidative stress, greatly enhanced β-alanine release in adult, as well as old patients[3]. In dementia of the Alzheimer's type, there is a significant decrease in the ratio between cerebrospinal fluid and plasma levels for alanine[4]. β-alanine (250-500 mg/kg) significantly reduced pilocarpine-induced tremors in rats[5]. It showed potent antineoplastic effect in mice[6].

Glycine significantly improved retrieval of information in both young and middle-aged adults, without however, affecting attention[7]. Glycine and β-alanine act mainly in the spinal cord and in the brain stem, via the strychnine sensitive glycine receptor. Glycine exhibits also a key rule in the excitatory neurotransmission in the N-methyl-D-aspartate (NMDA) receptor complex[8]. Loss of glutamatergic function appears to be responsible for the deposition of amyloid beta-peptide plaques, found in the brains of individuals suffering from Alzheimer's disease[9]. The NMDA receptor complex may be looked upon as the biological target for modulating learning and memory processes. D-serine may function as an endogenous agonist of the glycine site on the NMDA receptor, that has been implicated in the pathophysiology of Alzheimer's disease[10]. Levels of the neurotransmitter acetylcholine have been described as abnormally low in patients with Alzheimer's disease. These low levels of acetylcholine are presumed to be related to the memory loss. Thus, one of the treatments of Alzheimer's disease is based on to reverse decreased acetylcholine[11]. In the light of the above observations, the present study was undertaken to assess the potential of β-alanine as a memory-enhancing agent, employing elevated plus-maze and passive avoidance models of learning and memory in mice.

MATERIALS AND METHODS

Swiss male albino mice, younger ones (3 months old and weighing around 25-30 g) and aged ones (around 16 months old and weighing around 30-40 g), were used. Animals were procured from the Disease Free Small

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Animal House, CCS Haryana Agricultural University, Hisar (Haryana). They were acclimatized to the laboratory conditions for 5 days before behavioral studies. The animals had free access to food and water, and were housed under standard light-dark cycle (12 h each). All experiments were carried out during day time from 0900 to 1500 h. Institutional Animals Ethics Committee (IAEC) approved the experimental protocol (CPCSEA registration no. 436).

β-alanine (Hi-Media, Mumbai, India) and scopolamine hydrobromide (Sigma-Aldrich, USA) were dissolved in normal saline. Diazepam injection (Calmpose® Ranbaxy Labs., Gurgaon) and ethanol absolute were diluted with normal saline. All drugs were injected intraperitoneally (i.p.). Volume of injection was 1 ml/100 g body weight of the mouse. The following experimental models were used for studying learning and memory.

Elevated plus-maze:

Elevated plus-maze was originally introduced as a model for studying anxiolytic agents. Later on, it was found that acquisition and retention processes of memory could also be studied using elevated plus-maze. However, the parameters used for testing these two categories of agents were distinctly different. The procedure and end-point applied in the present study for testing learning and memory, was as per the criteria described by the investigators working in the area of psychopharmacology and behavioral pharmacology[12-18]. Briefly, the apparatus consisted of two open arms (16 cm × 5 cm), and two covered arms (16 cm × 5 cm × 12 cm). The arms extended from a central platform (5 cm × 5 cm), and the maze was elevated to a height of 25 cm from the floor. On the first day, each mouse was placed at the end of an open arm, facing away from the central platform. Transfer-latency (TL) was taken as the time taken by the mouse to move into one of the covered arms with all its four legs. TL was recorded on the first day. Cut-off time observed was 90-seconds. The mouse was allowed to explore the maze for another 10- seconds, and then returned to its home cage. Memory retention was examined 24-h after the first day trial, that is, on the 2nd day.

Passive-avoidance apparatus:

It was another sensitive-model employed in the present study, for testing learning and memory. This apparatus[13,15,17,18] comprised of a box ($27 \times 27 \times 27$ cm^3) having three walls of wood and one wall of plexiglass, featuring a grid-floor (3 mm stainless steel rods set 8 mm apart), with a wooden platform ($10 \times 7 \times 1.7$ cm^3) in the center of the grid-floor. Electric shock of 20V-AC was delivered to the grid-floor. The box was illuminated with a 15-W bulb during the experimental period. Training was carried out in two similar sessions. Each mouse was gently placed on the wooden-platform, set in the center of the grid-floor. When the mouse stepped down and placed all its paws on the grid-floor, shocks were delivered for 15-seconds, and step-down-latency (SDL) was recorded. SDL was defined as the time taken by the mouse to step down from wooden-platform to the grid-floor, with all its paws on the grid-floor. Animals showing SDL in the range of 2-15 seconds during the first test, were used for the second session and the retention test. The second-session was carried out 90-min. after the first test. When the animals stepped down before 60-seconds, electric shocks were delivered for 15 sec. During the second test, animals were removed from the shock-free-zone, if they did not step down for a period of 60 seconds. Retention was tested after 24-h in a similar manner, except that the electric shocks were not applied to the grid-floor. Each mouse was again placed on the platform, and the SDL was recorded with an upper cut-off time of 300-seconds. Increase in step-down latency (SDL) time is considered as evidence for successful acquisition, and retention of unpleasant experience.

The animals were divided into following 34 groups. Each group comprised of a minimum of 5 animals.

Using elevated plus maze:

Young mice:

Group I: Normal saline was injected i.p. for 6-days. TL was noted after 60 min. of injection of normal saline on the 6th day, and again after 24-h, i.e. on the 7th day. Group II and III: Beta-alanine (10 and 20 mg/kg i.p. respectively) was injected for 3-days to young mice. TL was noted after 60 min. of injection on the 3rd day, and again after 24-h, i.e. on 4th day. Group IV, V, VI and VII: β-alanine (5, 10, 20 and 40 mg/kg i.p. respectively), was injected for 6-days to young mice. TL was noted after 60 min. of injection on the 6th day, and again after 24-h, i.e. on the 7th day. Group VIII: Scopolamine hydrobromide (0.4 mg/kg i.p.) was injected to young mice before exposing the animals to plus maze test (training). TL was noted 45 min,and 24-hours after injection. Group IX: β-alanine (10 mg/kg i.p.) was injected to young mice for 6 days. After 60 min. of injection on the 6th day, scopolamine hydrobromide (0.4 mg/kg) was injected i.p. TL was noted after 45 min of injection of scopolamine, and again after 24 h, i.e. on the 7th day. Group X: Transfer latency was recorded on first day. On the

second day (after 24 h), Scopolamine hydrobromide (0.4 mg/kg i.p.) was injected 45 min prior to exposing the animals to plus maze (retention test). Group XI: β-alanine (10 mg/kg) was injected i.p. for 6 successive days. TL was noted after 60 min of administration on the 6th day. Scopolamine hydrobromide (0.4 mg/kg) was injected on the 7th day, 45 min prior to recording TL. Group XII: Diazepam (1.0 mg/kg) was injected i.p. TL was noted 45 min, and 24-hours after injection. Group XIII: β-alanine (10 mg/kg) was injected i.p. to young mice for 6 days. After 60 min. of injection of β-alanine on 6th day, diazepam (1.0 mg/kg) was injected i.p. TL was noted after 45 min of injection of diazepam, and again after 24-h, i.e. on the 7th day. Group XIV: Ethanol (1.0 g/kg) was injected i.p. before exposing the animals to plus maze test (training). Transfer latency was recorded after 45 min of injection, and again after 24- h. Group XV: Transfer latency was recorded on first day. On the second day (after 24 h), Ethanol (1.0 g/kg i.p.) was injected 45 min prior to exposing the animals to plus maze (retention test). Group XVI: β-alanine (10 mg/kg i.p.) was administered for 6 days. Ethanol (1.0 g/kg) was injected i.p. after 60 min of administration of β-alanine, on the 6th day. TL was recorded after 45 min of injection of ethanol, and again after 24 hours. Group XVII: β-alanine (10 mg/kg i.p.) was administered for 6 days. TL was recorded after 60 min of administration of β-alanine on the 6th day. After 24 h (that is on 7th day), ethanol (1.0 g/kg) was injected 45 min prior to exposing the animals to plus maze (retention test).

Aged mice:

Group XVIII: The vehicle (normal saline) was administered i.p. for 6 successive days to aged- mice. TL was recorded after 60 min of administration on the 6th day, and again after 24 h. Group XIX and XX: β-alanine (10 and 20 mg/kg respectively) was administered i.p. for 6 successive days to aged-mice. TL was recorded after 60 min of administration on the 6th day, and again after 24 h.

Using passive avoidance paradigm:

Young mice:

Group XXI: Normal saline (1 ml/100 g) was administered i.p. for 6 successive days, to young mice. SDL was recorded after 60 min of administration on the 6th day, and again after 24-h. Group XXII and XXIII: β-alanine (10 and 20 mg/kg i.p. respectively) was injected for 6-days to young mice. The last injection was given 60 min. before experimentation. SDL was noted on the 6th day, and again on 7th day. Group XXIV: SDL was recorded during 1st and 2nd training sessions on first day. Scopolamine hydrobromide (0.4 mg/kg) was injected i.p. 45 min prior to exposing the animals to passive avoidance apparatus, and retention was tested after 24 h. Group XXV: β-alanine (10 mg/kg i.p.) was injected i.p. to young mice for 6-days. SDL for the two training sessions was recorded after 60 min of injection on the 6th day. Scopolamine hydrobromide (0.4 mg/kg) was injected i.p. 45 min prior to recording SDL on the 7th day. Group XXVI: SDL was recorded in 1st and 2nd training sessions, on first day. Diazepam (1.0 mg/kg) was injected i.p. 45 min prior to exposing the animals to passive avoidance apparatus, and retention was tested after 24 h. Group XXVII: β-alanine (10 mg/kg i.p.) was injected i.p. to young mice for 6-days. SDL was recorded after 60 min of injection for the two training sessions. Diazepam (1.0 mg/kg) was injected i.p. 45 min prior to recording SDL on the 7th day. Group XXVIII: SDL was recorded during the 1st and 2nd training sessions, on the first day. Ethanol (1.0 g/kg) was injected i.p. 45 min prior to exposing the animals to passive avoidance apparatus, and retention was tested after 24 h. Group XXIX: β-alanine (10 mg/kg i.p.) was injected i.p. to young mice for 6-days. SDL for the two training sessions was recorded after 60 min of injection on the 6th day. Ethanol (1.0 g/kg) was injected i.p. 45 min prior to recording SDL on the 7th day.

Aged mice:

Group XXX: Normal saline (1 ml /100g) was administered i.p. for 6-days, to aged-mice**.** SDL was recorded after 60 min of administration on the 6th day, and again after 24-h. Group XXXI and XXXII: β-alanine (10 and 20 mg/kg i.p.) was injected for 6-days to aged-mice. The last injection was given 60 min before experimentation. SDL was noted on the 6th day, and again on the 7th day.

Locomotor activity:

Group XXXIII and XXXIV: Effect of β-alanine (20 mg/kg i.p. administered for 6 successive days) on locomotor function of young and aged mice respectively, was studied using Photoactometer (INCO, Ambala, India), to rule out the increase in locomotor performance of mice due to β-alanine treatment. The difference in the locomotor activity scores were noted before and after β-alanine treatment.

Statistical analysis:

All results were expressed as mean ± standard error of mean (SEM). All the groups were analyzed using Kruskal - Wallis ANOVA. For comparison of two groups, Mann-Whitney U-test was applied. The data for locomotor activity scores was subjected to student paired t-test. In all the tests, the criterion for statistical significance was $P<0.05$.

RESULTS

Using photoactometer:

There was no significant effect (P>0.05) on the locomotor activity of young (211 ± 25.3) or aged mice (201.5 ± 14.5), when treated with 20 mg/kg of β-alanine for 6-successive days, as compared to control values (164.6 ± 18.4 and 253.8 ± 19.4 respectively).

Using elevated plus-maze:

There was no significant effect on transfer latencies (TL) of young mice treated with β-alanine (10 and 20 mg/kg i.p.) for 3 successive days, as compared to control group in retention test. On the other hand, β-alanine (10 and 20 mg/kg i.p.) injected for 6 consecutive days to young mice significantly decreased transfer latencies, as compared to their control group in retention test, indicating significant improvement of memory. On the other hand, lower dose (5 mg/kg) and highest dose (40 mg/kg) of β-alanine for 6 consecutive days, did not have any significant effect on transfer latencies of mice, as compared to control group in retention test (Table 1). Scopolamine hydrobromide (0.4 mg/kg) and diazepam (1.0 mg/kg) injected before training, significantly increased transfer latency on the 1st day and on the 2nd day, indicating significant impairment of learning and memory. Ethanol (1.0 g/kg) injected before training, significantly increased transfer latency on the 1st day only. On the other hand, scopolamine hydrobromide (0.4 mg/kg) and ethanol (1.0 g/kg) injected before retention, significantly increased transfer latency on the 2nd day, as compared to the control group, indicating significant impairment of memory.

β-alanine (10 mg/kg) for 6 consecutive days, significantly reduced the amnesia induced by scopolamine injected before training, on the 6th day and after 24 h, that is on the 7th day, as indicated by decreased TL, when compared to the respective scopolamine-treated group. This dose of β-alanine also significantly reversed the amnesia induced by scopolamine injected before retention, on the 7th day. β-alanine (10 mg/kg) for 6 consecutive days, significantly reversed the amnesia induced by diazepam, as indicated by decrease in TL, as compared to the diazepam-treated group. β-alanine (10 mg/kg) for 6 consecutive days, significantly reversed the amnesia induced by ethanol injected before training, or before retention, as indicated by decrease in TL, as compared to respective ethanol treated group (Table 2). There was significant increase in transfer latencies of aged mice, as compared to the control group for young mice, on the first exposure to elevated plus maze. β-alanine (10 mg/kg) for 6 successive days, significantly decreased transfer latencies of aged mice, as compared to their control group, on first exposure to elevated plus maze. On the other hand, higher dose of β-alanine (20 mg/kg), injected for 6 consecutive days to aged mice, significantly decreased transfer latencies on first exposure to plus maze, and after 24 h as compared to their respective control group, indicating significant improvement of memory (Table 3).

Using passive-avoidance paradigm:

β-alanine (10 and 20 mg/kg i.p.) administered for 6-successive days to young and aged mice, significantly increased step-down latency (SDL), when exposed to passive avoidance apparatus on the 7th day, as compared to their respective control groups, indicating significant improvement of memory. Ageing, scopolamine, diazepam, and ethanol, significantly decreased SDL, as compared to the control group for young mice, indicating significant impairment of memory. Furthermore, β-alanine (10 mg/kg) injected for 6 successive days, reversed scopolamine, diazepam, and ethanol-induced amnesia, as indicated by increased SDL, as compared to their respective control groups (Table 4).

DISCUSSION

The findings of the present study revealed that β-alanine improved memory of both young and aged-mice, when

TABLE 1: EFFECT OF BETA-ALANINE ON TRANSFER-LATENCY (TL) OF YOUNG MICE USING ELEVATED PLUS-MAZE

Group No.	Treatment	Dose (kg^{-1})	TL on last day of treatment (s)	TL after 24- h (s)
I	Normal saline (Control)	10 ml	22.3±1.36	15.4±1.11
II	Beta-alanine for 3-days	10 mg	16.4±2.2	11.6±2.28
III	Beta-alanine for 3-days	20 mg	20.9±1.34	12.4±1.5
IV	Beta-alanine for 6-days	5 mg	24.9±3.66	18.98±3.12
V	Beta-alanine for 6-days	10 mg	9.36±1.86*	7.98±1.67*
VI	Beta-alanine for 6 -days	20 mg	12.3±0.97*	7.28±1.57*
VII	Beta-alanine for 6-days	40 mg	26.7±7.5	20.3±3.96

n = 5 in each group, Values are in Mean ± S.E.M, Kruskal-Wallis ANOVA, H (6) = 17.74, *P*<0.05 (last day of treatment), H (6) = 15.88, *P*<0.05 (after 24 h). *indicates *P*<0.05 as compared to control group (Mann-Whitney U-test)

TABLE 2: REVERSAL OF MEMORY DEFICITS INDUCED BY SCOPOLAMINE, DIAZEPAM OR ETHANOL BY BETA-ALANINE IN YOUNG MICE USING ELEVATED PLUS-MAZE

Group No.	Treatment	Dose (kg^{-1})	TL on last day of treatment (s)	TL after 24- h (s)
I	Normal saline (Control)	10 ml	22.3±1.36	15.4±1.11
VIII	Scopolamine (before training)	0.4 mg	52.2±6.3*	30.4±6.6*
IX	Beta-alanine 6-days + Scopolamine (before training)	10 mg 0.4 mg	17.3±1.77[a]	9.66±1.58[a]
X	Scopolamine (before retention)	0.4 mg	20.7±1.95	40.6±5*
XI	Beta-alanine 6-days + Scopolamine (before retention)	10 mg 0.4 mg	15.1±1.79	16.04 ±4[a]
XII	Diazepam (before training)	1 mg	49.7±7.03*	30.4±6.79*
XIII	Beta-alanine for 6-days + Diazepam (before training)	10 mg 1 mg	18.1±2.26[a]	12.8±1.5[a]
XIV	Ethanol (before training)	1 g	36.8±2.9*	14.8±2.7
XV	Beta-alanine for 6 days + ethanol (before training)	10 mg 1 g	9.9±1.45[a]	8.74±1.18
XVI	Ethanol (before retention)	1 g	25.6±0.5	37.5±1.1*
XVII	Beta-alanine for 6 days + ethanol (before retention)	10 mg 1 g	11.6±0.93[a]	11.4±1.2[a]

n = 5 in each group, Values are in Mean ± S.E.M, Kruskal-Wallis ANOVA H (10) = 47.505, $P<0.05$ (last day of treatment), H (10) = 40.325, $P<0.05$ (after 24 h). *indicates $P<0.05$ as compared to control group (Mann-Whitney U-test). [a]indicates $P<0.05$ as compared to scopolamine, diazepam or ethanol alone (Mann-Whitney U-test).

TABLE 3: EFFECT OF BETA-ALANINE ON TRANSFER-LATENCY (TL) OF AGED-MICE USING ELEVATED PLUS-MAZE

Group No.	Treatment	Dose (kg^{-1})	TL on last day of treatment (s)	TL after 24h (s)
I	Control (young mice)	10 ml	22.3±1.4	15.4±1.1
XVIII	Control (aged-mice)	10 ml	36.4±0.88*	16.2±1.47
XIX	Beta-alaninefor 6-days	10 mg	15±1.45[a]	15.8±0.77
XX	Beta-alaninefor 6-days	20 mg	12.9±0.74[a]	7.66±0.45[a]

n = 5 in each group, Kruskal-Wallis ANOVA H (3) = 16.25, $P<0.05$ (last day of treatment), H (3) = 10.96, $P<0.05$ (after 24 h). *indicates $P<0.05$ as compared to control group for young mice (Mann-Whitney U-test), [a]indicates $P<0.05$ as compared to control group for aged-mice (Mann-Whitney U-test).

TABLE 4: EFFECT OF BETA-ALANINE ON STEP-DOWN LATENCY (SDL) OF MICE USING PASSIVE-AVOIDANCE APPARATUS

Group No.	Mice	Treatment	Dose (kg^{-1})	SDL after24-h (s)
XXI	Young	Control (Saline)	10 ml	135.6±13.4
XXII	Young	Beta-alanine for 6-days	10 mg	209.6±20.8*
XXIII	Young	Beta-alanine for 6-days	20 mg	197.4±14.3*
XXIV	Young	Scopolamine	0.4 mg	15.6±2.21 *
XXV	Young	Beta-alanine for 6-days + Scopolamine	10 mg 0.4 mg	29.8±3.46[a]
XXVI	Young	Diazepam	1.0 mg	35.4±4.15*
XXVII	Young	Beta-alanine for 6-days + Diazepam	10 mg 1.0 mg	229.9±33.8[a]
XXVIII	Young	Ethanol	1.0 g	21.2±3.44*
XXIX	Young	Beta-alanine for 6-days + Ethanol	10 mg 1.0 g	219.2±34.7[a]
XXX	Aged	Control (Saline)	10 ml	81.6±7.4*
XXXI	Aged	Beta-alanine for 6-days	10 mg	202.8±40.6[b]
XXXII	Aged	Beta-alanine for 6-days	20 mg	273±13.2[b]

n = 5 in each group, Values are in Mean ± S.E.M, Kruskal – Wallis ANOVA: H (11) = 50.52; $P<0.05$, *indicates $P<0.05$ as compared to control group for young mice (Mann-Whitney U – test). [a]$P<0.05$ as compared to scopolamine, diazepam or ethanol alone (Mann-Whitney U – test), [b]$P<0.05$ as compared to control group for aged-mice (Mann-Whitney U – test)

tested on passive-avoidance and elevated plus-maze paradigms. Since, there was no significant effect on locomotor activity of young or aged mice treated with β-alanine, the improvement of memory due to β-alanine treatment, appears to be independent of locomotor performance of mice. Passive avoidance paradigm was capable of revealing age- related memory impairment more clearly, than elevated plus maze. Ageing, scopolamine, diazepam and ethanol, all significantly decreased SDL, as compared to the control group for young mice, indicating significant impairment of memory. Furthermore, β-alanine (10 mg/kg) injected for 6 successive days, significantly reversed amnesia produced by scopolamine (anti-cholinergic agent), ethanol (GABA agonist), diazepam, and ageing, as indicated by increased SDL, as compared to their respective control groups, in

the present study. The central cholinergic pathways play a prominent role in the learning and memory processes[19,20]. The decline in cognitive function is predominantly related to the decrease in cholinergic neurotransmission, and the degree of cholinergic neurodegeneration correlates positively with severity of memory impairment[21]. Since β-alanine reversed scopolamine-induced amnesia, it is likely that β-alanine improved memory, perhaps by increasing acetylcholine levels in the brain. Benzodiazepine induced amnesia appears to be mediated through benzodiazepine receptors, since flumazenil (benzodiazepine receptor antagonist) and beta-carbolines (benzodiazepine inverse agonist) reversed benzodiazepine-induced amnesia[22]. A role for GABA (gamma amino butyric acid) in the memory enhancing activity of β-alanine might explain the reversal of ethanol- induced amnesia by β-alanine observed in the present study. This is substantiated by the findings of Zarrindast *et al*, 2002 wherein, activation of GABA receptors have been shown to impair memory[23].

Normal ageing is known to deteriorate memory in human beings. In the present study, aged-animals showed impaired memory due to ageing process. Oxygen-free radicals are implicated in the process of ageing, and may be responsible for the development of Alzheimer's disease in elderly persons[24]. Antioxidant-rich diets improved cerebellar physiology and motor learning in aged-rats[25]. Furthermore, β-alanine has also been reported to possess antioxidant property[26] by virtue of which susceptible brain cells get exposed to less oxidative stress, resulting in reduced brain damage and improved neuronal function, thereby enhancing the memory. β-alanine also provided protection against toxic effects of the neurotoxin β-amyloid (the main culprit for impaired neurotransmission), on rat brain vascular endothelial cells[27].

Thus, it may be concluded that β-alanine has shown a promising memory- enhancing effect in both young and aged mice. Although, the exact mechanism of memory strengthening effect of β-alanine was not explored in the present study, it appears to be related to a combination of a variety of its properties such as antioxidant, anti-amyloid, and procholinergic.

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