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Differential Effects of Methylphenidate on Locus Coeruleus Neurons and Behavior Across Developmental Stages

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ABSTRACT

The increasing use of Methylphenidate (MPD) among adolescents and adults raises important concerns regarding its developmental and long-term neurophysiological effects. This study examined behavioral and neuronal responses in the Locus Coeruleus (LC)—a critical hub for Norepinephrine (NE) signaling—following administration of various MPD doses in adolescent and adult rats. Four experimental groups were used for each age cohort: saline, 0.6, 2.5, and 10.0 mg/kg MPD. Animals received six consecutive daily MPD injections from Experimental Days (ED) 1 to 6, followed by a three-day washout period and a single rechallenge dose on ED10. A total of 174 adolescent and 157 adult rats were used for concurrent behavioral assessments, while LC neuronal activity was recorded from 409 and 461 neurons, respectively. Significant age-dependent differences emerged in both acute and chronic responses to MPD. As the MPD dose increased, a greater number of rats and LC neurons exhibited altered activity. Notably, some animals displayed behavioral and neuronal sensitization at all MPD doses, whereas others exhibited tolerance. These effects varied significantly between age and MPD groups in both magnitude and incidence. Furthermore, Baseline (BL) LC activity following the six-day MPD regimen and after the washout period (ED10) was markedly altered, particularly in adolescents, indicating withdrawal-related changes. These shifts in BL activity from ED1 to ED10 indicate the presence of withdrawal behavior. This study highlights clear age-dependent differences in response to chronic MPD exposure. The observed patterns of sensitization, tolerance, and withdrawal serve as potential experimental biomarkers for drug dependence, emphasizing the clinical relevance of these findings. Importantly, the results underscore the value of combining electrophysiological and behavioral analyses and reaffirm the central role of the LC and NE signaling in mediating MPD's effects, with distinct implications for adolescent and adult populations

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Introduction

Methylphenidate (MPD) is the most prescribed drug to treat behavioral disorders in adolescents and adults and its medical use is safe [1-10]. In recent years its use has increased in schools, colleges, and workplaces to improve performance, increase concentration and attention, enhance retrieval of information from memory, enhance cognitive abilities and energy, enhance physical activity and reduce mental and physical fatigue while increasing arousal and concentration levels, treat hyperactivity disorders, depression, awareness deficiency, and sleep disorders, help to reduce symptoms of inattention, hyperactivity and impulsivity, improve mood, foster feelings of wellbeing and improve alertness and concentration [3,6,7,11-15]. This surge of MPD use among healthy adolescents and adults has raised concern about its use in individuals without ADHD while the neurophysiological mechanism of its action remains elusive [6,7,16-24].

Nestled within the posterior area of the upper dorsolateral pons, adjacent to the lateral floor of the fourth ventricle, lies the Locus Coeruleus (LC), named for its distinct blue color [25]. Serving as the primary hub for the synthesis of norepinephrine (NE) within the brain [26]. The LC neuronal population is primarily composed of medium-sized neurons, their blue coloration owing to the presence of melanin granules [27]. The LC functions as a key homeostatic control center for the body [28,29]. Efferent connections of the LC extend from the medial prefrontal cortex, thalamic nuclei, nucleus paragigantocellularis, nucleus prepositus, and hypothalamic nuclei, facilitating the integration of autonomic and environmental stimuli, gaze control, and various other functions [28,30,31]. A vast network of afferent projections extends from the LC, predominantly utilizing NE as their neurotransmitter, intricately linking the LC to diverse regions of the nervous system, including the spinal cord, brainstem, cerebellum, hypothalamus, thalamic nuclei, amygdala, hippocampus, tectum, ventral tegmental area, and cortex. These projections exert excitatory influences across these regions to mediate arousal [25,32].

The LC-Norepinephrine (NE) system orchestrates the modulation of cortical, subcortical, cerebellar, brainstem, and spinal cord circuits, thereby exerting profound effects on a wide array of functions including arousal, the sleep-wake cycle, attention, memory, cognitive flexibility, creativity, emotional regulation, stress response, decision-making, and neuroplasticity [28,33]. Dysregulation within this system has been implicated in a spectrum of behavioral disorders, encompassing affective disorders, anxiety disorders, Post-Traumatic Stress Disorder (PTSD), Attention-Deficit/Hyperactivity Disorder (ADHD), and Alzheimer's disease.

The LC is part of the Reticular Activating System (RAS), alongside the reticular formation and raphe nuclei. It primarily functions through the transmission of NE via a g protein-coupled receptor pathway. Arousal, sleep-wake cycle, memory, emotions, and stress are all influenced by the LC, thereby impacting various neuropsychiatric disorders. Norepinephrine released from the LC acts on noradrenergic receptors (alpha and beta receptors) present on neurons and glial cells via adenylyl cyclase and phospholipase C pathways, thereby intricately regulating various physiological and cognitive processes within the central nervous system [31, 34]. In sum, the LC stands as a linchpin within the neural architecture, wielding profound influence over an extensive array of physiological and cognitive functions. Its intricate interplay with the broader neurocircuitry underscores its indispensability in maintaining homeostasis and orchestrating adaptive responses to the ever-changing milieu of internal and external stimuli. Therefore, the role of Methylphenidate (MPD) in adolescent and adult animals was investigated.

Dopamine (DA), Serotonin (SER) and Norepinephrine (NE) are participating in the pharmacotherapy action of MPD [18,19,20,35,36]. However, the main research focus on MPD property appears to focus on its effect on the DA system [37-40]. Research on the role of MPD on the noradrenergic system is limited. MPD bind also to Norepinephrine (NE) Transporter (NET) at the synaptic cleft [41-43] and prevents the reuptake of NE from the synaptic cleft back to the presynaptic terminals thus prolong the NE effects [43,44]. The increased and prolonged effect of NE in the locus coeruleus (LC) and other brain areas results in the improvement of attention, maintain alertness, arousal, concentration, and more [3,13,45,46] and account for MPD therapeutic and rewarding effects and the rationale of its used [39,40].

Recently, the use of MPD by adolescent and adult increase dramatically advocate to study the role of MPD in adolescent as compared to adult The objective of this study is to test the following hypotheses:

- The chronic administration of 0.6, 2.5, or 10.0 mg/kg of MPD will lead to behavioral and neuronal sensitization in some animals, while others receiving the same MPD dose will exhibit behavioral and neurophysiological tolerance when compared to the initial effects of the drug for each dose [7,47-49].
- Neuronal activity recorded from animals displaying behavioral sensitization to chronic MPD exposure will primarily show excitation in response to MPD treatment. In contrast, neuronal activity recorded from animals expressing behavioral tolerance will demonstrate mainly a decrease in firing rate in response to chronic MPD treatment compared to the acute (initial) effects of MPD.
- Adolescent animals will respond differently to 0.6, 2.5, or 10.0 mg/kg of MPD compared to adult animals. Specifically, the ratio of LC neurons responding to each MPD dose with excitation or attenuation will differ significantly between age groups, indicating an age-related difference in response to MPD which is clinically important to know for safe used of MPD. Part of the data recorded from adolescent rats only were reported (20), as well part of the data recorded from adult rats only were reported [32].

Result

Locomotor Behavior, Time and Saline (Control)

To assess the impact of time (growth over days), injection volume, and animal handling on locomotor behavioral activity, we used two groups of rats at each age: a time control group (N=8) receiving no injection, and no handling, and a saline (injection and handling) control group (N=8) for both adolescent and adult rats (N=12). Locomotor activity, including the number of movements (NOM), Total Distance Traveled (TD) in centimeters, and the number of stereotypic movements (NOS), was recorded over eleven consecutive days for both adolescent and adult rats in the un-injected time control group. These recordings indicated consistent levels of locomotor activity across all the experimental days.

Similarly, in the saline control group, locomotor activity recording showed no significant differences across recording days, with only minor, non-significant fluctuations. Following six consecutive daily saline injections and a subsequent three-day washout period (ED7-9), there was no notable change in locomotor activity at the post-washout baseline (ED7-9 BL) compared to the initial ED1 BL. Additionally, a saline rechallenge on ED10 did not significantly alter locomotor activity from the initial baseline (ED10 BL/ED1 BL). These findings indicate that locomotor activity measures (TD, NOM, and NOS) remained unaffected by the duration of the 10-day experimental period, the injection process, handling, or laboratory conditions [32].

Given the lack of significant differences in daily locomotor activity between the time control and saline control groups, the locomotor activity recorded on ED1 after saline injection was designated as the control baseline (ED1 BL) for comparisons with the MPD injection groups. Thus, any significant changes in locomotor activity from the ED1 BL can be attributed to MPD exposure effects [32].

MPD Injection Groups

Three hundred and thirtyone male Sprague-Dawley rats were used to simultaneously recording behavioral expression and Locus Coeruleus (LC) neuronal activity before and after acute and chronic MPD administration. The study included 174 adolescent and 157 adult rats. The adolescent group was subdivided into the following categories: time control (8 rats), acute and repetitive saline control injections (8 rats), and various doses of MPD: 0.6 mg/kg (52 rats), 2.5 mg/kg (49 rats), and 10.0 mg/kg (57 rats). The adult group was similarly divided into time control (12 rats), acute and repetitive saline control injections (12 rats), and MPD doses of 0.6 mg/kg (44 rats), 2.5 mg/kg (47 rats), and 10.0 mg/kg (42 rats).

Impact of Acute and Chronic 0.6 mg/kg MPD on Behavioral Activity of All Animals (Figure 1 All)

Fifty-two adolescent and 44 adult rats received acute and chronic injections of 0.6 mg/kg MPD. Both age groups exhibited a significant ($p<0.05$) change in locomotor activity following the initial administration of 0.6 mg/kg MPD on ED1 compared to the initial BL (ED1 MPD/ED1 BL) the adolescent exhibited significant ($p<0.05$) acceleration and the adult animals exhibit significant ($p<0.05$) attenuation (Figure 1 All). After six days of MPD exposure and a three-day washout period, there was a significant ($p<0.05$) reduction in BL activity on ED10 compared to ED1 (ED10 BL/ED1 BL). Upon rechallenge with MPD on ED10, no significant differences in locomotor activity were observed compared to the initial MPD response on ED1 (ED10 MPD/ED1 MPD).

Impact of Acute and Chronic 0.6 mg/kg MPD on Behavioral Sensitization (Figure 1 Sensitized)

Thirty-one adolescent rats out of 52 (31/52) 56.9% and 30/44 adult rats (68.2%) exhibited significant ($p<0.05$) behavioral sensitization. Both age groups showed significant ($p<0.05$) increases in locomotor

activity following acute MPD administration on ED1 (ED1 MPD/ ED1 BL). Notably, adolescent rats in the sensitized group displayed a significant ($p<0.05$) decrease in activity on ED10 compared to ED1 (ED10 BL/ED1 BL), while adult animals exhibit significant ($p<0.05$) increase in ED10 BL/ED1 BL using ANOVA. The Chi-square test confirmed a significant (χ^2 (10,532) =631.25; $p<0.05$) difference in sensitization rates between the two age groups in respond to MPD.

Impact of Acute and Chronic 0.6 mg/kg MPD on Behavioral Tolerance (Figure 1 Tolerant)

Behavioral tolerance to repetitive 0.6 mg/kg MPD administration was observed in 21/52 (40.4%) adolescent rats and 14/44 (31.8%) adult rats. Both age groups exhibit a significant ($p<0.05$) increase in activity after the initial MPD dose on ED1 (ED1 MPD/ED1 BL). Significant ($p<0.05$) changes were also observed in BL activity on ED10 compared to ED1 (ED10 BL/ED1 BL), with a significant ($p<0.05$) attenuation in activity on ED10 MPD/ ED1 MPD administration in both ag groups using the ANOVA. Behavioral tolerance was present in 21/52 of adolescent rats and in 16/44 of adult rats, with the Chi-square test revealing a significant (χ^2 (11,174) = 657.85; $p<0.05$) difference between the two groups in respond to MPD.

Impact of Acute and Chronic 2.5 mg/kg MPD on Behavioral Activity of All Animals (Figure 1 All)

Forty-nine adolescent and 47 adult rats received acute and chronic injections of 2.5 mg/kg MPD. Both groups showed a significant ($p<0.05$) increase in behavioral activity response following the initial MPD administration on ED1 (ED1 MPD/ED1 BL). After six days of MPD exposure and a three-day washout period, BL activity on ED10 BL/ED1 BL significantly ($p<0.05$) increased in adolescent rats, while no significant change was observed in adult rats (ED10 BL/ED1 BL). Upon rechallenge with MPD on ED10, both age groups exhibited significantly ($p<0.05$) increased behavioral activity responses compared to their initial responses on ED1 (ED10 MPD/ED1 MPD).

Impact of Acute and Chronic 2.5 mg/kg MPD on Behavioral Sensitization (Figure 1 Sensitized)

Twenty-eight/49 adolescent (57.1%) and 34/47 adult rats (72.3%) exhibited behavioral sensitization to a 2.5 mg/kg dose of MPD. Both age groups showed a significant ($p<0.05$) increase in locomotor behavioral activity following acute MPD administration on ED1 MPD/ED1 BL. Notably, only the adolescent rats displayed a significant ($p<0.05$) increase in BL activity on ED10 BL/ED1 BL after six days of MPD exposure and three washout days. Upon MPD rechallenge on ED10, both adolescents and adults showed significantly ($p<0.05$) heightened behavioral responses compared to their responses on ED1 (ED10 MPD/ED1 MPD). The proportion of adolescent rats demonstrating behavioral sensitization to 2.5 mg/kg MPD was (28 /49), whereas 34/47) of adult rats showed sensitization. The Chi-square test revealing a significant (χ^2 (10,333) =683.80; $p<0.01$) difference between the two age groups.

Impact of Acute and Chronic 2.5 mg/kg MPD on Behavioral Tolerance (Figure 1 Tolerant)

Behavioral tolerance to chronic MPD exposure was observed in 21/49 adolescent (42.9%) and 13/47 adult rats (27.7%) at a 2.5 mg/kg dose group. Both groups experienced a significant ($p<0.05$) increase in locomotor activity following acute MPD administration on ED1 (ED1 MPD/ED1 BL). However, the ED10 activity recorded from adolescent rats showed a significant ($p<0.05$) decrease in BL activity (ED10 BL/ED1 BL), while adults exhibited a significant increase ($p < 0.05$). Upon rechallenge with MPD on ED10, both age groups demonstrated significant ($p<0.05$) reductions in locomotor activity compared to their activity on ED1 (ED10 MPD/ED1 MPD) using two-way ANOVA, indicating the development of behavioral tolerance. The proportion of adolescent rats showing behavioral tolerance to 2.5 mg/kg MPD was 21/49), compared to 13/47 in adult rats. The Chi-square test revealing a significant (χ^2 (11,532) = 683.80; $p<0.001$) difference between the two age groups in response to MPD.

Impact of Acute and Chronic 10.0 mg/kg MPD on Behavioral Activity of All Groups (Figure 1 All)

The effects of both acute and chronic administration of 10.0 mg/kg MPD were assessed in 57 adolescent and 42 adult rats (Figure 3 All). Both groups showed a significant ($p<0.05$) increase in behavioral activity following acute MPD administration on ED1 MPD/ED1 BL. After six days of MPD exposure, both groups exhibited a significant ($p<0.05$) rise in locomotor activity from their ED1 BL to ED10 BL (ED10 BL/ED1 BL). However, the increase in activity was significantly ($p<0.05$) greater in adolescent rats compared to adults. When rechallenged with MPD on ED10, both groups demonstrated a significant ($p<0.05$) increase in their behavioral response (ED10 MPD/ED1 MPD) using two-way ANOVA.

Impact of Acute and Chronic 10.0 mg/kg MPD on Behavioral Sensitization (Figure 1 Sensitized).

Behavioral sensitization to chronic 10.0 mg/kg MPD exposure was evident in 33/57 (57.9%) adolescent rats and 13/42 (31.0%) adult rats. Following the acute administration of 10.0 mg/kg MPD on ED1, both groups exhibited significant ($p<0.05$) increases in locomotor activity (ED1 MPD/ED1 BL). On ED10, only the adolescent rats showed a significant ($p<0.05$) increase in BL activity compared to ED1 (ED10 BL/ED1 BL). Upon rechallenge with MPD on ED10, both age groups exhibited further significant ($p<0.05$) increased their locomotor activity (ED10 MPD/ED1 MPD) using two-way ANOVA, indicating behavioral sensitization. The proportion of adolescent rats exhibiting behavioral sensitization to 10.0 mg/kg MPD was 33/57), while 13/42) of adult rats showed sensitization. The Chi-square test revealing a significant (χ^2 (11,911) =730.83; $p<0.01$) difference between the two age groups in response to MPD.

Impact of Acute and Chronic 10.0 mg/kg MPD on Behavioral Tolerance (Figure 1 Tolerance).

Behavioral tolerance to chronic 10.0 mg/kg MPD was observed in 24/57 (42.1%) adolescent and 29/42 (69.0%) adult rats. Both age groups displayed significant ($p<0.05$) increases in locomotor activity following acute MPD administration on ED1 MPD/ED1 BL, with adults showing significantly ($p<0.05$) more activity than adolescents using two-way ANOVA. Only the adolescent group showed a significant ($p<0.05$) increase in ED10 BL/ED1 BL, indicating potential withdrawal effects. Upon MPD rechallenge on ED10, both groups exhibited significant ($p<0.05$) decreases in locomotor activity (ED10 MPD/ED1 MPD), demonstrating behavioral tolerance. The proportion of adolescent rats showing tolerance to 10.0 mg/kg MPD was 24/57), while 29/42 of adult rats showed tolerance. The Chi-square test revealing a significant (χ^2 (9, 337) =603.13; $p<0.05$) difference between the two age groups in response to MPD.

Comparison of Number of Movements (NOM) Between All, Sensitized, and Tolerant Groups

A comparison of behavioral responses across different MPD doses (0.6, 2.5, and 10 mg/kg) in adolescent and adult rats to acute MPD

on ED1 (ED1 MPD/ED1 BL), changes in BL activity in ED10 BL/ED1 BL after six days of MPD administration and three days of washout, and the effects of chronic MPD exposure ED10 MPD/ED1 MPD were also significantly ($p<0.05$) different between the two age groups using two-way ANOVA. The Chi-square test revealing a significant (χ^2 (12,377) =757.38; $p<0.001$) difference between the two age groups in response to MPD.

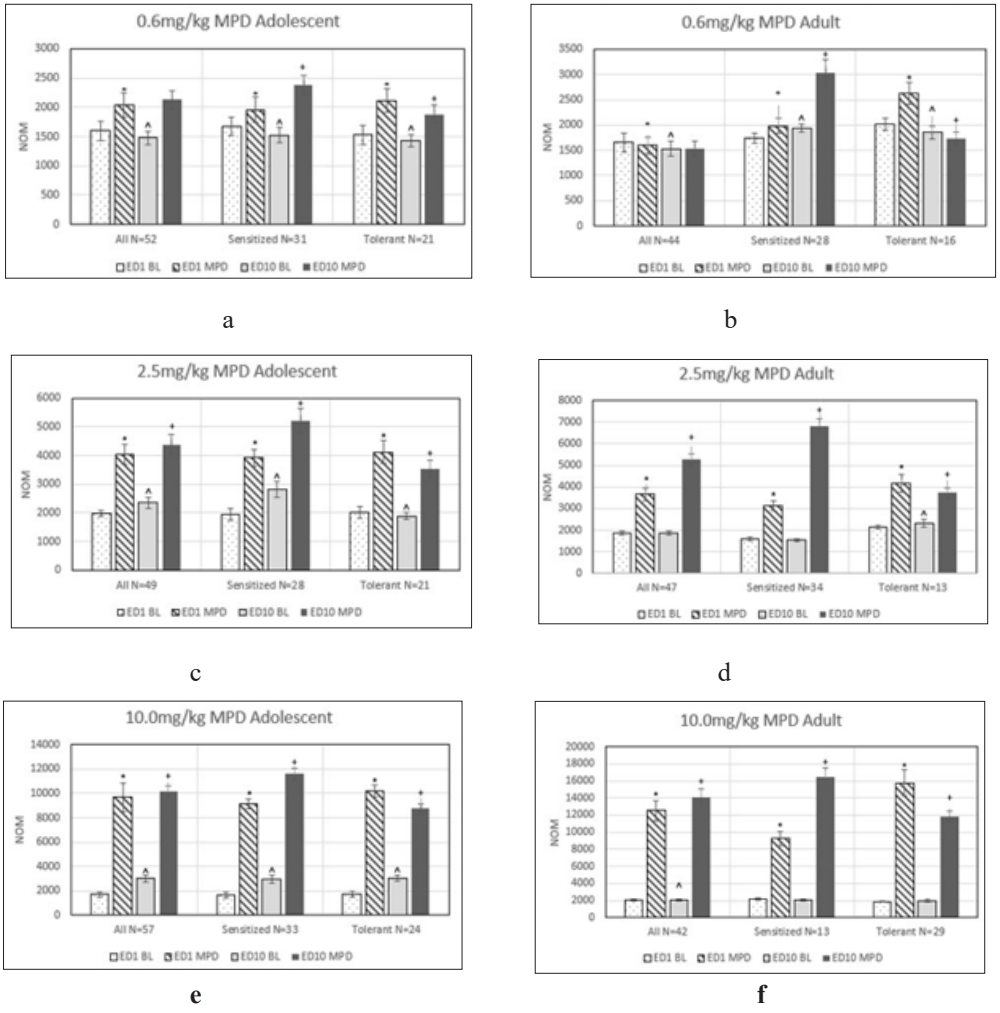

Figure 1: This figure summarizes behavioral data (Number of Movements, NOM) for adolescent and adult rat groups. Panels A and B: Saline control. Panels C-F: Each experimental MPD dose (0.6, 2.5, and 10.0 mg/kg) for adolescent (left) and adult (right) rats of the three groups (all, sensitized, tolerant). N represents the number of animals in each group. Each histogram contains four columns: ED1 BL, ED1 MPD, ED10 BL, and ED10 MPD, organized into three comparisons per subgroup: ED1 MPD/ED1 BL (acute effect), ED10 BL/ED1 BL (effect of six daily MPD exposures and three washout days on ED10 BL), and ED10 MPD/ED1 MPD (chronic MPD effect). The figure shows NOM comparisons between adolescent and adult for ED1 BL, ED1 MPD, ED10 BL, and ED10 MPD. Each column shows the standard deviation (SD). * Indicates significant ($p < 0.05$) differences from ED1 BL (acute). Δ Indicates significant ($p < 0.05$) differences from ED1 BL to ED10 BL (withdrawal). ‡ Indicates significant ($p < 0.05$) differences from ED1 MPD (chronic).

Table 1 – LC Adolescent

A	**All Animals**										
	Dose	**N**	**Acute (ED 1 MPD/ED 1 BL)**			**Baseline (ED 10 BL/ED 1 BL)**			**Chronic (ED 10 MPD/ED 1 MPD)**		
			↑	↓	-	↑	↓	-	↑	↓	-
	Saline	56	1 (2%)	1 (2%)	54 (96%)	2 (4%)	2 (4%)	52 (92%)	2 (4%)	1 (2%)	53 (94%)
	0.6 mg/kg	109	21 (19%)	37 (34%)	51 (47%)	21 (19%)	34 (31%)	54 (50%)	37 (34%)	20 (18%)	52 (48%)
	2.5 mg/kg	132	52 (39%)	29 (22%)	51 (39%)	59 (45%)	40 (30%)	33 (25%)	55 (42%)	35 (27%)	42 (31%)
	10.0 mg/kg	112	89 (79%)	13 (12%)	10 (9%)	85 (76%)	14 (12%)	14 (12%)	75 (67%)	29 (26%)	8 (7%)
	Total	409									
B	Sensitized										
	Dose	N	Acute (ED 1 MPD/ED 1 BL)			Baseline (ED 10 BL/ED 1 BL)			Chronic (ED 10 MPD/ED 1 MPD)		
			↑	↓	-	↑	↓	-	↑	↓	-
	0.6 mg/kg	47	35 (74%)	5 (11%)	7 (15%)	15 (32%)	13 (28%)	19 (40%)	38 (81%)	6 (13%)	3 (6%)
	2.5 mg/kg	61	37 (61%)	16 (26%)	8 (13%)	36 (59%)	12 (20%)	13 (21%)	35 (57%)	15 (25%)	11 (18%)
	10.0 mg/kg	81	59 (72%)	11 (14%)	11 (14%)	63 (78%)	9 (11%)	9 (11%)	56 (69%)	19 (24%)	6 (7%)
	Total	189									
	Tolerance										
C	Dose	N	Acute (ED 1 MPD/ED 1 BL)			Baseline (ED 10 BL/ED 1 BL)			Chronic (ED 10 MPD/ED 1 MPD)		
			↑	↓	-	↑	↓	-	↑	↓	-
	0.6 mg/kg	62	12 (19%)	19 (31%)	31 (50%)	12 (19%)	31 (34%)	29 (34%)	14 (23%)	17 (27%)	3129 (50%)
	2.5 mg/kg	71	24 (34%)	17 (24%)	30 (43%)	24 (34%)	32 (45%)	15 (21%)	21 (30%)	29 (40%)	21 (30%)
	10.0 mg/kg	31	26 (84%)	3 (10%)	2 (6%)	21 (68%)	7 (22%)	3 (10%)	12 (39%)	17 (55%)	2 (6%)
	Total	164									

Table 1: The table summarizes the Locus Coeruleus (LC) neuronal responses following 0.6, 2.5, and 10.0 mg/kg MPD in adolescent animals. Sections A, B, and C provide summaries from all animals (All group, 2A), animals showing behavioral sensitization (Sensitized group, 2B), and animals showing behavioral tolerance (Tolerance group, 2C), respectively. Under "Acute," "Baseline," and "Chronic," the table indicates the number and percentage (in brackets) of LC neurons responding significantly ($p<0.05$) to each MPD dose by excitation (↑), attenuation (↓), and non-response (-) following acute MPD (ED1 MPD/ED1 BL). It compares the baseline activity on ED10 to ED1 (ED10 BL/ED1 BL) and the chronic MPD effect (ED10 MPD/ED1 MPD).

Table 2 – LC Adult

A	All Animals										
	Dose	N	Acute (ED 1 MPD/ED 1 BL)			Baseline (ED 10 BL/ED 1 BL)			Chronic (ED 10 MPD/ED 1 MPD)		
			↑	↓	-	↑	↓	-	↑	↓	-
	Saline	56	1 (2%)	2 (4%)	53 (94%)	3 (5%)	3 (5%)	50 (91.2%)	2 (4%)	1 (2%)	53 (94%)
	0.6 mg/kg	128	29 (23%)	21 (16%)	78 (61%)	47 (37%)	67 (52%)	14 (11%)	43 (34%)	63 (49%)	22 (17%)
	2.5 mg/kg	136	81 (60%)	25 (18%)	30 (22%)	51 (37%)	81 (60%)	4 (3%)	54 (40%)	71 (52%)	11 (8%)
	10.0 mg/kg	141	93 (66%)	46 (33%)	2 (1%)	62 (44%)	77 (55%)	2 (1%)	53 (38%)	53 (38%)	3 (2%)
	Total	461									
	Sensitized										
B	Dose	N	Acute (ED 1 MPD/ED 1 BL)			Baseline (ED 10 BL/ED 1 BL)			Chronic (ED 10 MPD/ED 1 MPD)		
			↑	↓	-	↑	↓	-	↑	↓	-
	0.6 mg/kg	66	23 (35%)	10 (15%)	33 (50%)	27 (41%)	14 (21%)	25 (38%)	42 (64%)	3 (4%)	21 (32%)
	2.5 mg/kg	65	32 (49%)	16 (25%)	17 (26%)	36 (55%)	16 (25%)	13 (20%)	39 (60%)	15 (23%)	11 (17%)
	10.0 mg/kg	96	73 (76%)	16 (17%)	7 (7%)	63 (78%)	24 (25%)	9 (9%)	78 (81%)	8 (8%)	10 (11%)
	Total	227									
	Tolerance										
C	0.6 mg/kg	71	16 (23%)	38 (52%)	18 (25%)	21 (30%)	21 (30%)	29 (40%)	14 (20%)	26 (37%)	31 (43%)
	2.5 mg/kg	93	39 (42%)	20 (22%)	34 (36%)	40 (43%)	33 (36%)	20 (21%)	22 (24%)	40 (43%)	31 (33%)
	10.0 mg/kg	99	85 (86%)	11 (11%)	3 (3%)	81 (82%)	13 (13%)	5 (5%)	16 (16%)	81 (82%)	2 (2%)
	Total	265									

Table 2: This table summarizes the LC neuronal responses following 0.6, 2.5, and 10.0 mg/kg MPD in adult animals. Sections A, B, and C provide summaries from all animals (3A), animals showing behavioral sensitization (3B), and animals showing behavioral tolerance (3C), respectively. Under "Acute," "Baseline," and "Chronic," the table indicates the number and percentage (in brackets) of LC neurons responding significantly ($p<0.05$) to each MPD dose by excitation (↑), attenuation (↓), and non-response (-) following acute MPD (ED1 MPD/ED1 BL). It compares the baseline activity on ED10 to ED1 (ED10 BL/ED1 BL) and the chronic MPD effect (ED10 MPD/ED1 MPD).

Locus Coeruleus (LC) Neuronal Activity (Tables 1,2)

A total of 870 LC neurons were recorded: 409 from adolescent rats and 461 from adult rats. All recordings were histologically confirmed to originate from the LC nucleus. The neuronal recordings exhibited similar waveform spikes and amplitudes on ED1 compared to ED10. Figure 3 show typical Pre-Frontal Cortex (PFC) neuronal activity and figure 4 show representative histogram of PFC neuronal histogram before and after acute and chronic MPD exposure.

Control Recordings (Tables 1A and 2A saline)

Fifty-six LC neurons were recorded from adolescent rats, and an additional 56 from adult rats, serving as controls following acute and repetitive saline injections (Table 1A and 2A). Among the adolescent rats: On ED1, 1 out of 56 (2%) LC neurons showed a significant change in activity, and another 1/56 (2%) showed a decrease in activity, while 54/56 (96%) showed no change (ED1 Sal/ED1 BL). By ED10, after six saline injections and three washout days, 2/56 (4%) neurons showed increased activity, 2/56 (4%) showed decreased activity, and 52/56 (92%) showed no change (ED10 BL/ED1 BL). Upon saline rechallenge on ED10, 2/56 (4%) neurons showed increased activity, 1/56 (2%) showed decreased activity, and 53/56 (94%) showed no change (ED10 Sal/ED1 Sal). For the adult rats: On ED1, 1/56 (2%) neuron showed increased activity, 2/56 (4%) showed decreased activity, and 53/56 (94%) showed no change (ED1 Sal/ED1 BL). On ED10, 3/56 (5%) neurons showed increased activity, 3/56 (5%) showed decreased activity, and 50/56 (90%) showed no change (ED10 BL/ED1 BL). Upon saline rechallenge on ED10, 2/56 (4%) neurons showed increased activity, 1/56 (2%) showed decreased activity, and 53/56 (94%) showed no change (ED10 Sal/ED1 Sal). These observations suggest that changes in baseline (BL) activity following saline injection were random, indicating that the saline injections, animal handling, and laboratory conditions did not significantly affect LC neuronal activity in both age groups over the experimental days. Therefore, significant changes in neuronal recordings from ED1 saline (ED1 BL) are due to MPD exposure.

Impact of Acute and Chronic 0.6 mg/kg MPD on LC Neurons Recorded from All Animals (Tables 1A & 2A, 0.6mg/kg)

In adolescent animals, a total of 109 LC neurons were recorded following acute and repetitive (chronic) administration of 0.6 mg/kg

MPD. On ED1, 58/109 (53%) neurons showed a significant ($p<0.05$) change in firing rate, with the majority 37 out of 58 (64%) showing a significant ($p<0.05$) decrease in ED1 MPD compared to ED1 BL (ED1 MPD/ED1 BL). By ED10, after six daily MPD injections and three washout days, 55/109 (50%) neurons showed significant ($p<0.05$) changes, with 34/55 (62%) showing ($p<0.05$) decreased activity (ED10 BL/ED1 BL). Upon MPD rechallenge on ED10, 57/109 (52%) neurons showed significant ($p<0.05$) changes, with 37/57 (65%) showing significant ($p<0.05$) increased activity (ED10 MPD/ED1 MPD).

In adult animals, a total of 128 LC neurons were recorded. On ED1, 50/128 (39%) neurons showed significant ($p<0.05$) changes, with 29/50 (58%) showing significant ($p<0.05$) increased firing rates (ED1 MPD/ED1 BL). By ED10, 114/128 (90%) neurons showed significant ($p<0.05$) changes in their firing rate, with 67/114 (59%) showing significant ($p<0.05$) decreased activity (ED10 BL/ED1 BL). Upon MPD rechallenge on ED10, 106/128 (83%) neurons showed significant ($p<0.05$) changes, with 63/106 (59%) showing significant ($p<0.05$) decreased activity (ED10 MPD/ED1 MPD).

Impact of Acute and Chronic 0.6 mg/kg MPD on LC Neurons Recorded from Behavioral Sensitization Animals (Tables 1B and 2B)

Forty-seven LC neurons were recorded in adolescent animals exhibiting behavioral sensitization to chronic MPD. On ED1, 40/47 (85%) LC neurons showed significant ($p<0.05$) changes in their firing rate, with 35/40 (88%) showing significant($p<0.05$) increased firing rates (ED1 MPD/ED1 BL). By ED10, 28/47 (60%) neurons showed significant ($p<0.05$) changes, with 15/28 (54%) showing significant ($p<0.05$) increased activity (ED10 BL/ED1 BL). Upon MPD rechallenge on ED10, 44/47 (94%) neurons showed significant ($p<0.05$) changes, with 38/44 (86%) showing significant ($p<0.05$) increased activity (ED10 MPD/ED1 MPD).

sixty-six LC neurons were recorded in adult animals. On ED1, 33/66 (50%) neurons showed significant ($p<0.05$) changes, with 23/33 (70%) showing significant ($p<0.05$) increased firing rates (ED1 MPD/ED1 BL). By ED10, after six daily MPD and three washout days, 41/66 (62%) LC neurons showed significant ($p<0.05$) changes in their ED10 BL/ED1 BL, with 27/41 (66%) showing significant ($p<0.05$) increased activity (ED10 BL/ED1 BL). Upon MPD rechallenge on ED10, 45/66 (68%) LC neurons showed significant ($p<0.05$) changes, with 42/66 (64%) showing significant ($p<0.05$) increased activity (ED10 MPD/ED1 MPD).

Impact of Acute and Chronic 0.6 mg/kg MPD on LC Neurons Recorded from Behavioral Tolerance Animals (Tables 1C and 2C)

Sixty-two LC neurons were recorded in adolescent animals demonstrating behavioral tolerance. On ED1, 31/62 (50%) LC neurons showed significant ($p<0.05$) changes, with 19/31 (61%) showing significant ($p<0.05$) decreased firing rates (ED1 MPD/ED1 BL). By ED10, after six daily MPD exposure and three washout days, 33/62 (53%) neurons showed significant ($p<0.05$) changes in their firing rate, with 21/33 (64%) showing significant ($p<0.05$) decreased activity (ED10 BL/ED1 BL). Upon MPD rechallenge on ED10, 31/62 (50%) LC neurons showed significant ($p>0.05$) changes, with 17/31 (55%) showing significant ($p<0.05$) decreased activity (ED10 MPD/ED1 MPD).

In adult animals, 71 LC neurons were recorded. On ED1, 53/71 (75%) neurons showed significant ($p<0.05$) changes, with 37/ (70%) showing significant ($p<0.05$) decreased firing rates (ED1 MPD/ED1 BL). By ED10, 42/71 (59%) neurons showed significant ($p<0.05$) changes, with 21/42 (50%) showing significant ($p<0.05$) increased activity and 21/42 (50%) showing significant ($p<0.05$) decreased activity (ED10 BL/ED1 BL). Upon MPD rechallenge on ED10, 40/71 (56%) neurons showed significant ($p<0.05$) changes, with 26/40 (65%) showing significant ($p>0.05$) decreased activity (ED10 MPD/ED1 MPD).

Impact of Acute and Chronic 2.5mg/kg MPD on LC neurons recorded from All animals (Tables 1A & 2A)

A total of 132 LC neuronal activities were recorded from adolescent rats following the administration of 2.5 mg/kg MPD. Among these, 81/132 neurons (62%) showed a significant ($p<0.05$) change in firing rate at ED1 MPD/ED1 BL, with the majority (52/81, 64%) exhibiting a significant ($p<0.05$) increase in firing rate, 99/132 neurons (75%) displayed a significant ($p<0.05$) change in firing rate at ED10 BL/ED1 BL, with 59/99 (60%) showing a significant ($p<0.05$) increase. Following MPD rechallenge at ED10 MPD/ED1 MPD, 90/132 neurons (68%) responded with a significant ($p<0.05$) change in activity, with 55/90 (61%) showing a significant ($p<0.05$) increase (Table 1A, 2.5 mg/kg MPD All).

In adult animals, 136 LC neurons were recorded. Of these, 106/136 neurons (80%) responded with a significant ($p<0.05$) change in firing activity following acute MPD administration, with 81/106 neurons (60%) showing an increase in firing rate. Comparing ED10 BL/ED1 BL, 132/136 neurons (97%) exhibited a significant ($p<0.05$) change in firing rate after six days of MPD exposure and three washout days, with 81/132 neurons (61%) showing a significant ($p<0.05$) decrease in firing rate. Upon MPD rechallenge at ED10 MPD/ED1 MPD, 125/136 neurons (92%) responded with a significant ($p<0.05$) change in firing rate, with 71 neurons (57%) showing a significant ($p<0.05$) decrease (Table 2A, 2.5 mg/kg MPD All).

Impact of Acute and Chronic 2.5 mg/kg MPD on LC neurons recorded from Behavioral Sensitization animals (Tables 1B and 2B)

Sixty-one LC neurons were recorded from adolescent animals demonstrating behavioral sensitization to chronic exposure of 2.5 mg/kg MPD (Table 2B). At ED1 MPD/ED1 BL, 53/61 neurons (87%) showed a significant ($p<0.05$) change in firing rate, with 37/53 (70%) showing a significant ($p<0.05$) increase. At ED10 BL/ED1 BL, 48/61 neurons (78%) exhibited a significantly ($p<0.05$) different firing rate, with 36/48 (75%) showing a significant ($p<0.05$) increase. Upon rechallenge at ED10 MPD/ED1 MPD, 50/61 neurons (82%) showed a significant ($p<0.05$) change in firing rate, with 35/50 (70%) showing a significant ($p<0.05$) increase (Table 1B, 2.5 mg/kg MPD, Sensitized).

Sixty-five LC neurons were recorded from adult animals demonstrating behavioral sensitization to chronic exposure of 2.5 mg/kg MPD (Table 2B). At ED1 MPD/ED1 BL, 48/65 neurons (74%) showed a significant ($p<0.05$) change in firing rate, with 32/48 (67%) showing a significant ($p<0.05$) increase. At ED10 BL/ED1 BL, 52/65 neurons (80%) exhibited a significant ($p<0.05$) change in firing rate, with 36/52 (69%) showing a significant increase. Upon rechallenge at ED10 MPD/ED1 MPD, 54/65 neurons (83%) showed a significant ($p<0.05$) change in firing rate, with 39/54 (72%) showing a significant ($p<0.05$) increase (Table 2B, 2.5 mg/kg MPD, Sensitized).

Impact of Acute and Chronic 2.5 mg/kg MPD on LC neurons recorded from Behavioral Tolerance animals (Tables 1C and 2C)

Seventy-one LC neurons were recorded from adolescent animals demonstrating behavioral tolerance to chronic 2.5 mg/kg MPD (Table 1C). At ED1 MPD/ED1 BL, 41/71 neurons (58%) showed

a significant ($p<0.05$) change in firing rate, with 24/41 (59%) showing an increase. At ED10 BL/ED1 BL, 56/71 neurons (79%) exhibited a significantly ($p<0.05$) different firing rate, with 32/56 (57%) showing a significant ($p<0.05$) decrease. Upon rechallenge at ED10 MPD/ED1 MPD, 50/71 neurons (70%) showed a significant ($p<0.05$) change in firing rate, with 29/50 (58%) showing a significant ($p<0.05$) decrease (Table 1C, 2.5 mg/kg, Tolerance).

Ninety-three LC neurons were recorded from adult animals demonstrating behavioral tolerance to chronic 2.5 mg/kg MPD (Table 2C). At ED1 MPD/ED1 BL, 59/93 neurons (64%) showed a significant ($p<0.05$) change in firing rate, with 39/59 (66%) showing an increase. At ED10 BL/ED1 BL, 73/93 neurons (78%) exhibited a significant ($p<0.05$) change in firing rate, with 40/73 (55%) showing a significant ($p<0.05$) increase. Upon rechallenge at ED10 MPD/ED1 MPD, 62/93 neurons (67%) showed a significant ($p<0.05$) change in firing rate, with 40/62 (65%) showing a significant ($p<0.05$) decrease (Table 2C, 2.5 mg/kg MPD, Tolerance).

Impact of Acute and Chronic 10.0 mg/kg MPD on LC Neurons Recorded from All Animals (Tables 1A & 2A)

A total of 112 LC neurons were recorded following administration of 10 mg/kg MPD in adolescent animals (Table 1A). Of these neurons, 102/112 (91%) exhibited a significant ($p<0.05$) change in neuronal firing rate, with 89/102 (87%) showing a significant increase ($p<0.05$) in firing rate. After six days of MPD exposure (ED10 BL) and three washout days, 99/112 (88%) neurons showed a significant change ($p<0.05$), with 85/99 (86%) exhibiting an increased baseline firing rate (ED1 BL). Upon MPD rechallenge at ED10 MPD/ED1 MPD, 104/112 (93%) neurons responded with a significant change ($p<0.05$) in activity, with 75/104 (72%) showing a significant increase ($p<0.05$) in firing rate (Table 1A, 10 mg/kg MPD, All). In adult rats, 141 LC neurons were recorded. Among them, 139/141 (99%) showed a significant change ($p<0.05$) in firing rate at ED1 MPD/ED1 BL, with 93/139 (67%) demonstrating an increase. At ED10 BL/ED1 BL, 131/141 (99%) exhibited a significant change ($p<0.05$) in baseline activity, with 77/139 (55%) showing a significant ($p<0.05$) decrease. Upon rechallenge at ED10 MPD/ED1 MPD, 138/141 (98%) responded with a significant change ($p<0.05$), with 85/138 (62%) showing a decrease ($p<0.05$) in firing rate (Table 2A, 10 mg/kg MPD, All).

Impact of Acute and Chronic 10.0 mg/kg MPD on LC Neurons Recorded from Behavioral Sensitization Animals (Tables 1B and 2B)

Eighty-one LC neurons were recorded from adolescent animals demonstrating behavioral sensitization to chronic 10.0 mg/kg MPD (Table 1B). At ED1 MPD/ED1 BL, 70/81 (86%) showed a significant change ($p<0.05$) in firing rate, with 59/70 (84%) exhibiting an increase. At ED10 BL/ED1 BL, 72/81 (89%) demonstrated a significant change ($p<0.05$), with 63/72 (88%) showing an increase. Upon rechallenge at ED10 MPD/ED1 MPD, 75/81 (93%) responded with a significant change ($p<0.05$), with 56/75 (75%) showing an increase ($p<0.05$) in firing rate (Table 1B, 10 mg/kg MPD, Sensitization). In adult animals, 96 LC neurons were recorded. At ED1 MPD/ED1 BL, 89/96 (93%) showed a significant change ($p<0.05$) in firing rate, with 73/89 (82%) showing an increase. At ED10 BL/ED1 BL, 87/96 (91%) showed a significant change ($p<0.05$) in firing rate, with 63/87 (72%) showing an increase. Upon rechallenge at ED10 MPD/ED1 MPD, 86/96 (90%) responded with a significant ($p<0.05$) change, with 78/86 (91%) showing a significant increase ($p<0.05$) in firing rate (Table 2B, 10 mg/kg MPD, Sensitized).

Impact of Acute and Chronic 10.0 mg/kg MPD on LC Neurons Recorded from Behavioral Tolerance Animals (Tables 1C and 2C)

Thirty-one LC neurons were recorded from adolescent animals demonstrating significant ($p<0.05$) behavioral tolerance to chronic 10.0 mg/kg MPD (Table 1C). At ED1 MPD/ED1 BL, 29/31 (94%) showed a significant change ($p<0.05$) in firing rate, with 26/29 (90%) exhibiting an increase. At ED10 BL/ED1 BL, 28/31 (90%) displayed a significant change ($p<0.05$) in baseline activity, with 21/28 (75%) showing an increase. Upon rechallenge at ED10 MPD/ED1 MPD, 29/31 (94%) responded with a significant change ($p<0.05$) in firing rate, with 17/29 (59%) showing a decrease ($p<0.05$). In adult animals, 99 LC neurons were recorded. At ED1 MPD/ED1 BL, 96/99 (97%) showed a significant change ($p<0.05$) in firing rate, with 85/96 (89%) showing an increase. At ED10 BL/ED1 BL, 94/99 (95%) showed a significant change ($p<0.05$), with 81/94 (86%) showing an increase. Upon rechallenge at ED10 MPD/ED1 MPD, 97/99 (98%) responded with a significant change ($p<0.05$), with 81/97 (84%) showing a decrease ($p<0.05$) in firing rate (Table 2C, 10 mg/kg MPD, Tolerance).

Comparing adolescent LC Neuron Response Direction (Increase or Decrease) to MPD with adult LC Neurons response direction (Figure 2)

Figure 2 displays histograms comparing the percentage of LC neuronal responses directions (Increase or Decrease) to 0.6, 2.5, and 10.0 mg/kg MPD in adolescent animals (Fig 2 A, B & C) and adult animals (Fig 2 D, E & F). Upper Histograms (Fig 2 A & D): display the responsiveness direction for the All group. Middle Histograms (Fig 2 B & E): Display the responsiveness direction for the Sensitized groups. Lower Histograms: (Fig 2 C &F) Display the responsiveness direction for the Tolerance groups. The percentage of LC neurons recorded from acute MPD responding significantly ($p<0.05$) to acute 0.6, 2.5, or 10.0 mg/kg MPD (ED1 MPD/ED1 BL) is summarized on Fig 2A for adolescent and Fig 2D for adult. The percentage of LC neurons recorded a ED10 BL/ED1 BL after six daily MPD exposure and three washout days is summarized on Fig 2B for adolescent and Fig 2E for adult. The percentage of LC neurons recorded from chronic MPD responding significantly ($p<0.05$) to 0.6, 2.5, or 10.0 mg/kg MPD (ED10 MPD/ED1 MPD) is summarized on Fig 2C for adolescent and Fig 2F for adult respectively. Comparisons between the three adolescent groups (All, Sensitized and Tolerance) show significant differences (χ^2 (10,352) = 698; $p<0.01$) between the All, Sensitized, and Tolerant groups in response to the 0.6, 2.5, and 10.0 mg/kg MPD doses. Comparisons between the three adult groups (All, Sensitized and Tolerance) show significant differences (χ^2 10,053) = 691; $p<0.01$) between the All, Sensitized, and Tolerant groups in response to the 0.6, 2.5, and 10.0 mg/kg MPD doses. Neuronal responses recorded from behaviorally sensitized adolescent animals to 0.6 and 2.5 mg/kg MPD differ significantly (χ^2 (11,871) = 689.25; $p<0.001$) from those recorded from behaviorally tolerant animals or from the LC neurons recorded from the All group (Figure 2 left). Similar significant differences (χ^2 (11,329) = 662.75; $p<0.001$) are observed in the adult groups following 0.6 and 2.5mg/kg MPD (Figure 2 right). No significant age differences in response direction between adolescent and adult groups to acute and chronic 10.0 mg/kg MPD (Figure 2 lower histograms) were observed. These findings underscore the importance of evaluating neuronal recordings based on the animal's behavioral response to chronic drug exposure and using a dose-response protocol.

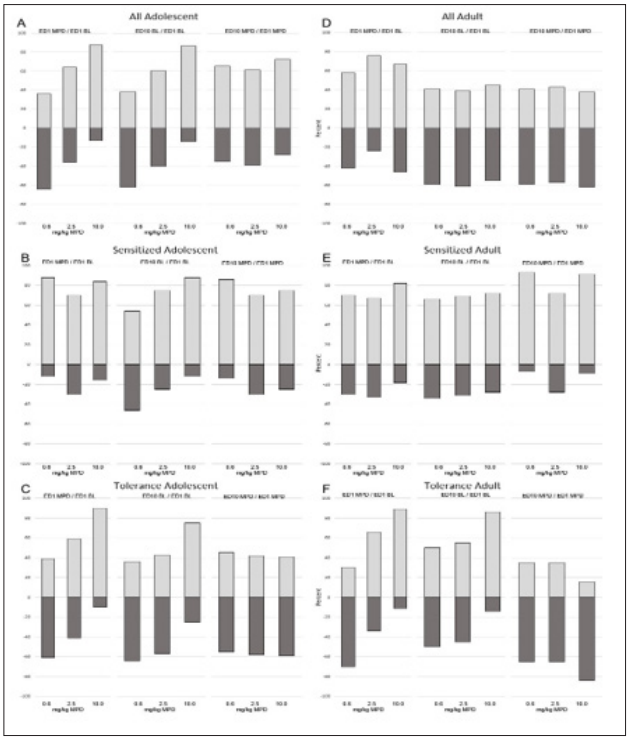

Figure 2: This figure summarizes the direction of responsiveness (% increase or decrease) of LC neurons to acute and chronic MPD doses. Sections A and D: LC neurons recorded following 0.6, 2.5, and 10.0 mg/kg MPD from all animals (All group). Sections B and E: LC neurons recorded following 0.6, 2.5, and 10.0 mg/kg MPD from the behavioral sensitized animals (Sensitized group). Sections C and F: LC neurons recorded following 0.6, 2.5, and 10.0 mg/kg MPD from the behavioral tolerant animals (Tolerance group) in adolescent and adult animal groups respectively. Each section shows the percentage of LC neurons responding significantly by either increasing or decreasing firing rates in response to: Acute MPD (ED1 MPD/ED1 BL); baseline change (ED10 BL/ED1 BL) after six daily MPD exposures and three washout days, and chronic MPD effect (ED10 MPD/ED1 MPD).

Discussion

The Locus Coeruleus (LC) noradrenergic neurons are the primary source of Norepinephrine (NE) for various brain regions (26), playing a vital role in regulating sensory network processing. These neurons are involved in several functions, including arousal, cognitive function, memory, sleep-wake cycle, attention, and emotion [46]. Methylphenidate (MPD) enhances noradrenergic transmission by blocking NE reuptake from the synaptic cleft back into the presynaptic terminal through binding to the NE Transporter (NET). This action increases and prolongs NE's effects in the postsynaptic domain [42,44]. Given the LC's central role in these processes, it was chosen for a study to investigate whether there are age (adolescent compared to adult) related similarities or differences in LC neuronal and animals' behavioral activity responses following both acute and chronic exposure to three different MPD doses (low, medium, and high).

Key Findings

The study's key findings include the following:

1. Both adolescent and adult animals showed either behavioral sensitization or tolerance to chronic MPD at doses of 0.6, 2.5, and 10.0 mg/kg.
2. The proportion of adolescent animals responding behaviorally to each MPD dose was significantly different from that of adult animals.
3. Individual animal responses behaviorally to chronic MPD doses differed significantly from the initial (acute) MPD effects. This variability was observed both within each age group and between the age groups.
4. Age differences in LC neuronal responses exhibit significantly differently, showing differences in excitation or attenuation to each MPD dose and between behaviorally sensitized and tolerant animals.
5. The total responsiveness and the proportion of LC neurons responding to each MPD dose was significantly aged different from that of adult animals.
6. The ratio how many LC neurons exhibiting excitation or attenuation in response to each MPD dose varied significantly between the two age groups.
7. Neuronal and behavioral responses to MPD increased proportionally with higher MPD doses in both age groups, indicating that the responses were dose-dependent.
8. There were significant differences between adolescents and adults in their LC neuronal and behavioral responses to chronic MPD. This included differences in the proportion of animals and LC neurons displaying behavioral sensitization or tolerance.
9. Chronic MPD exposure led to distinct electrophysiological changes recorded in the LC between adolescent and adult animals and between LC neurons from behaviorally sensitized and tolerant animals.
10. Recordings from behaviorally sensitized animals showed increased excitation following chronic MPD exposure compared to the initial MPD exposure. In contrast, recordings from behaviorally tolerant animals exhibited reduced in the initial excitation in response to repetitive (chronic) MPD exposure.
11. All the above significant age differences were observe following 0.6 and 2.5mg/kg MPD but following the MPD higher dose of 10.0mg/kg both age response similarly. The findings suggest age-related differently in MPD responses.

Possible Mechanisms

The proposed mechanism of MPD action includes the following:

1. **Neurotransmitter Modulation**
   Acute MPD exposure stimulates the release of NE, dopamine (DA), and serotonin (5-HT) and binds to their respective transporters, preventing their reuptake into presynaptic terminals (35, 36, 46, 50). This results in increased levels of these neurotransmitters in the synaptic cleft, leading to heightened LC neuronal and behavioral activities [24,3].
2. **Plasticity and Gene Expression**
   Repeated MPD exposure is associated with plasticity in the mesocorticolimbic system (51- 55). Changes in gene expression, controlled by transcription factors such as CREB and Δ Fos B, play a crucial role in this plasticity [53,56,57]. Following acute MPD exposure, adolescent and adult animals generally respond similarly. However, significant age differences in response to chronic MPD exposure were observed, particularly at doses of 0.6 and 2.5 mg/kg.
3. **Neuroplasticity in Adolescents**
   Age differences in response to MPD can be attributed to ongoing neuroplasticity processes in adolescent animals compared to adults. MPD's role in eliciting sensitization may involve extracellular signal-regulated kinase (ERK) phosphorylation, leading to increased levels of Δ Fos B, AMPA receptor subunits, and subsequent increases in

neuronal and behavioral activities [53,56,58-60]. In contrast, tolerant animals may exhibit upregulation of CREB, resulting in decreased AMPA receptor subunits and reduced neuronal and behavioral activities.

4. **Receptor Distribution Differences**
   Individual differences in NE, DA, and 5-HT receptor proportions within the LC and other brain areas may explain the varying responses to chronic MPD. Age-related changes in NE receptor distributions might contribute to the observed differences in excitation and attenuation between adolescent and adult animals [61].
5. **Neuronal Excitation-Inhibition Mechanism**
   Each brain site has neurons that response to a stimulus by excitation and other neurons response to the same stimulus by inhibition of the BL activity i.e., each brain site has both response mechanisms. The ratio of neurons responding to a stimulus (in our case MPD) with excitation versus attenuation in a specific brain region dictates its overall response of the structure to a given stimulus.

The findings highlight significant age-related differences in response to MPD, emphasizing the need to evaluate the drug's effects separately in adolescents and adults. The variability in LC neuronal and behavioral responses to chronic MPD—whether sensitization or tolerance—suggests the importance of individual animals' assessments. This is crucial, especially given the increasing prevalence of MPD use in adolescent populations. Sensitization and tolerance expression are considered experimental biomarkers for dependence and addiction [18,19,20,24,62-64]. The potential for MPD to cause dependence and addiction underscores the importance of tailored approaches to its use and monitoring in clinical settings.

Conclusion

In conclusion, chronic MPD exposure leads to complex and age-dependent neurophysiological and behavioral responses. These findings provide insights into the mechanisms underlying MPD sensitization and tolerance, highlighting the need for individualized evaluation strategies. The significant responses of LC neurons to acute and chronic MPD exposure indicate that NE and the LC play a crucial role in the response to MPD exposure.

Materials and Methods

Animals

Adolescent male Sprague-Dawley rats at post-natal day 30 (P-30) and adult at P-50 were purchased from Harlan (Indianapolis, IN, USA). The rats were allowed an acclimation period of 3-5 days, had ad libitum access to food and water. The animal room was maintained at a temperature of 21 ± 2°C with a relative humidity of 37-45%, under a 12-hour light/dark cycle, with lights on at 6:00 AM.

Surgery and Electrode Implantation

Rats were anesthetized with an Intraperitoneal (i.p) injection of pentobarbital dosed at 30 mg/kg for adolescents and 50 mg/kg for adults. Each rat's head was shaved, and lidocaine hydrochloride topical gel was applied to the shaved area for local anesthesia. The rats were then placed in a stereotaxic head holder. An incision was made to expose the skull by removing the skin and head muscles. Bilateral holes were drilled in the exposed skull above the Locus Coeruleus (LC) at 8.0 mm posterior to the bregma for adolescents and 10.4 mm for adults, and 1.2 mm and 1.5 mm lateral to the midline, with a depth of 7.0 mm and 7.2 mm, respectively, using the Sherwood and Timiras adolescent rat brain atlas for adolescent rats and the Paxinos and Watson Rat Brain Atlas for adult rats [65,66]. An additional hole was drilled in front of the frontal sinus for the reference electrode. Six anchor screws were inserted into vacant spots on the skull to secure the head cap with dental acrylic cement.

Two twisted Nickel-Chromium wires (insulated except at the tips) measuring 60 µm in diameter were secured to 1 cm copper connector pins and inserted into the LC in each hemisphere, resulting in four total recording electrodes per animal. Neuronal activity was monitored during electrode placement. Electrodes were secured to the skull with dental acrylic cement if sufficient neuronal activity was observed, demonstrating a 3:1 signal-to-noise ratio. If satisfactory activity was not detected, electrodes were lowered in increments of 5 to 10 µm until satisfactory spike activity was observed.

Post-Surgical Care and Acclimation

After electrode implantation, the animals were returned to their home cages. They were allowed an additional 5-7 days to recover before recording sessions commenced. All experimental procedures were approved by the University of Texas Health Science Center Animal Welfare Committee and were in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

During the 5-7 days recovery period from electrodes implantation, animals with their home cage were moved to the experimental room for two hours each day. They were connected to a wireless head stage (Triangle Biosystems Inc., TBSI, Durham, NC, USA) for neuronal activity monitoring and to the Accuscan behavioral system (Accuscan Com, Ohio USA) for the behavioral recording for adaptation and acclimation to the experimental procedure.

Experimental Protocol and Data Acquisition

Locus Coeruleus (LC) neuronal and locomotor behavioral activities recording of animals starting at postnatal day 40 (P-40) for adolescent rats and postnatal day 60 (P-60) for adult rats (Table 3). A wireless neuronal recording system (TBSI, Durham, NC, USA) in conjunction with an open field computerized animal activity system and OASIS data collection software (Accuscan, Columbus, OH, USA) were used and described in detailed previously [18, 19, 20, 24, 63, 64]. In sort, the TBSI head stage was connected to a rat head cap with electrode pins, transmitting wireless electrical signals to a remote receiver linked to an analog-to-digital converter (Micro 1401-3; Cambridge Electronic Design (CED England)). Neuronal activity from each electrode was recorded and stored on a PC using CED Spike 2.7 software. The open field system consisted of a 40x40x32 cm cage equipped with 16x16 infrared beams, which albino rats do not detect due to their insensitivity to red light. The infrared sensors were placed at heights of 5 and 8 cm above the cage floor. The Accuscan monitored animal movement system detecting interruptions in the infrared beams at a frequency of 100 Hz. The Oasis software (Accuscan, Columbus, OH, USA) compiled data on beam interruptions every 10 minutes and categorized them into three locomotive behaviors: number of movements (NOM), total distance traveled (TD) in cm, and number of stereotypic movements (NOS), defined as repetitive movements with at least one-second intervals between them. Data were recorded for 60 minutes post-injection of either saline or methylphenidate (MPD) on experimental day (ED) 1 and ED 10 (Table 3).

Treatment Groups		ED 1*	ED 2-6	ED 7-9	ED10*
1	Saline	Saline/Saline	Saline	Washout	Saline/Saline
2	0.6 mg/kg MPD	Saline/0.6 mg/kg MPD	0.6 mg/kg MPD	Washout	Saline/0.6 mg/kg MPD
3	2.5 mg/kg MPD	Saline/2.5 mg/kg MPD	2.5 mg/kg MPD	Washout	Saline/2.5 mg/kg MPD
4	10.0 mg/kg MPD	Saline/10.0 mg/kg MPD	10.0 mg/kg MPD	Washout	Saline/10.0 mg/kg MPD

Table 3: This table outlines the four animal groups and the MPD dosing protocol for adolescent animals. Four similar groups were used for adult animals. The groups for each age category are: saline, 0.6 mg/kg MPD, 2.5 mg/kg MPD, and 10.0 mg/kg MPD. On Experimental Day 1 (ED1), animals received an initial dose of saline, and recordings were taken for one hour to establish a baseline (ED1 BL). This was followed by one of the designated injections: saline, 0.6, 2.5, or 10.0 mg/kg of MPD, with recordings continued for an additional hour post-injection (i.e., ED1 MPD). On Experimental Days 2-6, animals received a morning injection of the designated dose. ED7-9 were washout days with no injections. On ED10, animals received another saline dose to establish the baseline (ED10 BL) after six days of injections and three washout days, followed by the designated MPD dose injection (ED10 MPD) and additional one-hour recordings as on ED1. * Indicates behavioral and neuronal recording day.

Behavioral recordings were essential to differentiate animals exhibiting behavioral sensitization from those animals showing behavioral tolerance following repeated (chronic) MPD exposure compared to the initial MPD effect [19,20,24, 63,64]. The behavioral locomotive data provided a basis for analyzing neuronal recordings. Therefore, data were categorized into three groups:

- Behavioral and LC neuronal recording data obtained from all animals,
- Data obtained only from animals expressing behavioral sensitization, and
- data obtained only from animals expressing behavioral tolerance.

Each age group was randomly subdivided into four treatment groups: saline (control), 0.6, 2.5, and 10.0 mg/kg MPD. On experimental day 1 (ED1), rats were placed in their home cage within a Faraday testing cage to minimize background noise. After connecting the wireless head stage to the electrode pins, animals acclimated for 20-30 minutes before recording began. Baseline (BL) neuronal and behavioral activity (ED1 BL) was recorded for one hour following an initial injection of 0.8 ml saline. The animals then received a second injection of either saline or MPD (0.6, 2.5, or 10.0 mg/kg), and neuronal and locomotive behavior was recorded for an additional hour post injection (ED1 Sal or ED1 MPD, Table 3).

From ED2 through ED6, animals received daily injections of either saline or MPD in their home cages without recording to elicit the chronic MPD effects. ED7 through ED9 were washout days with no injections. On ED10, animals received a 0.8 ml saline injection, and BL neuronal and behavioral activity (ED10 BL) was recorded for one hour. Subsequently, animals were rechallenged with either saline or MPD (0.6, 2.5, or 10.0 mg/kg), and an additional hour of recording was performed as on ED1 Table 3 [19,20,24,63,64].

Behavioral Data Analysis

The data acquired was used to make three comparisons:

- **Acute Effects of MPD**
  Locomotor activity after acute MPD administration on ED1 was compared to the BL recording post-saline administration on ED1 (ED1 MPD/ED1 BL) to determine the acute effects of MPD.
- **Changes in ED 10 BL**
  Locomotor activity post-saline injection on ED10 was compared to locomotor activity post-saline injection on ED1 (ED10 BL/ED1 BL) to obtained any significant changes in ED10 BL locomotor activity as result of six daily repetitive (chronic) MPD exposures and three washout days.
- **Chronic Effects of MPD**
  Locomotor activity after MPD administration on ED10 was compared to locomotor activity after MPD administration on ED1 (ED10 MPD/ED1 MPD) to determine the chronic effect of MPD and assess whether the animal displayed behavioral sensitization or tolerance. Animals exhibiting significantly increased locomotor activity after MPD rechallenge on ED10 compared to MPD administration on ED1 were considered to display behavioral sensitization. Conversely, those animals exhibiting a significant decrease in locomotor activity on ED10 MPD/ED1 MPD were considered to display behavioral tolerance. To determine the effect of MPD on individual animals, the student t-test and the Critical Ratio (CR) test were used. $C.R. = \frac{E-C}{\sqrt{E+C}} = \pm 1.96$ = $p < 0.05$. A C.R. test value above +1.96 indicated a significant increase in activity ($p < 0.05$), while a value below -1.96 indicated a significant ($p<0.05$) decrease. For the acute effect of MPD, E represents the activity count after MPD injection on ED1, and C represents the ED1 activity after saline (control) injection (ED1 MPD/ED1 BL). For the chronic effect, E represents activity following saline or MPD injection on ED10, and C represents activity after saline or MPD dose on ED1 (ED10 BL/ED1 BL and ED10 MPD/ED1 MPD). Individual rats were categorized as exhibiting either behavioral sensitization or tolerance based on their response mention above and summed to sensitized or tolerance group. A significant difference among the groups was determined using a two-way ANOVA, with $p < 0.05$ accepted as the minimal level of significance.

Neuronal Data Analysis

Upon completion of spike sorting, the data was exported into a spreadsheet to calculate the average neuronal firing rates for each treatment and produce a sequential firing rate histogram [18,19,20,63,64]. Statistical comparisons were made for each LC neuronal activity as follows:

- LC neuronal activity after the initial MPD exposure was compared to LC neuronal activity following saline administration (BL) on ED1 (ED1 MPD/ED1 BL) to determine the acute effect of MPD.
- LC BL neuronal activity following saline injection on ED10 prior to MPD injection was compared with LC BL neuronal activity on ED1 (ED10 BL/ED1 BL) to assess withdrawal activity after six days of daily MPD exposure and three washout days.
- LC neuronal activity after MPD administration on ED10 was compared to the activity following initial MPD on ED1 (ED10 MPD/ED1 MPD) to assess the chronic effect of the drug

(sensitization or tolerance). Significant changes in neuronal firing rate and direction of change (increase or decrease) for each LC neuron were determined using the student's t-test and the Critical Ratio (C.R.) test. A C.R. value above +1.96 indicated a significant ($P<0.05$) increase in activity, while a C.R. value below -1.96 indicated a significant ($P<0.05$) decrease in activity after MPD administration. Additionally, the neurophysiological data was summarized into three groups based on the animals' behavioral responses to each chronic dose of MPD treatment:

- **All group:** Electrophysiological data obtained from all animals.
- **Sensitized group:** Electrophysiological data obtained only from animals exhibiting behavioral sensitization.
- **Tolerance group:** Electrophysiological data obtained only from animals exhibiting behavioral tolerance (Tables 1 and 2). The mean values for these three groups, all, sensitized and tolerant, were exported from OASIS software and each subgroup was further analyzed using Analysis of Variance (ANOVA) with reported measures. In addition, a post hoc Tukey test was run to compare significant ($P<0.05$) behavioral changes observed between experimental days ED1 and ED10 within each group as well as the Chi-square test with $p<0.05$ consider significant using SPSS software version 21.

Histological Verification of Electrode Placement

Upon completion of recording, an overdose of sodium phenobarbital was administered. Animals were perfused intracardially with 10% formaldehyde solution containing 3% potassium ferrocyanide, and a 20 μA current was passed through the electrode pin for 20 seconds to create a small lesion at the recording sites in the LC. The brain was removed and preserved in 10% formalin for histological processing. The histological sections were used to verify the electrode placement in the LC by locating the lesion and the Prussian blue spot, using the Sherwood and Timiras, Rat Brain Atlas for adolescent rats and the Paxinos and Watson Rat Brain Atlas for adult rats [65,66].

Drug

Previous experiments with MPD dose-response protocols ranging from 0.1 to 40.0 mg/kg i.p, show that behavioral effects of MPD were observed at doses of 0.6 mg/kg and higher [18,19,20,63,64]. Therefore, MPD was administered at doses of 0.6, 2.5, and 10.0 mg/kg, representing low, moderate, and high experimental dosages, respectively. MPD was dissolved in a 0.9% isotonic saline solution for i.p injection. Control subjects received injections of 0.8 ml isotonic saline solution (0.9% NaCl). All MPD injections were adjusted to a volume of 0.8 ml with 0.9% saline to ensure consistent injection volumes across all animals. Methylphenidate hydrochloride (MPD) was donated by Mallinckrodt Inc, (St. Louise USA) [67].

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