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Behavioral impairments in rats with chronic epilepsy suggest comorbidity between epilepsy and attention deficit and hyperactivity disorder

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Abstract

Attention deficit/hyperactivity disorder (ADHD) is encountered among epilepsy patients at a significantly higher rate than in the general population. Mechanisms of epilepsy-ADHD comorbidity remain largely unknown. We investigated whether a model of chronic epilepsy in rats produces signs of ADHD, and thus, whether it can be used for studying mechanisms of this comorbidity. Epilepsy was induced in male Wistar rats via pilocarpine status epilepticus. Half of the animals exhibited chronic ADHD-like abnormalities, particularly increased impulsivity and diminished attention in the lateralized reaction time task. These impairments correlated with the suppressed noradrenergic transmission in locus coeruleus outputs. The other half of animals exhibited depressive behavior in the forced swimming test congruently with the diminished serotonergic transmission in raphe nucleus outputs. ADHD and depressive behavior appeared mutually exclusive. Therefore, pilocarpine model of epilepsy affords a system for reproducing and studying mechanisms of comorbidity between epilepsy and both ADHD and/or depression.

Keywords

Epilepsy; attention deficit and hyperactivity disorder; depression; norepinephrine; serotonin; lateralized reaction time task

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1. Introduction

Attention deficit/hyperactivity disorder (ADHD) represents one of the most common comorbidities of epilepsy: its prevalence among epilepsy patients is >20% as opposed to 5% in general population [1–4]. Although an epidemiological connection between epilepsy and ADHD is well established, mechanisms of the comorbidity (as well as mechanisms of ADHD as a stand-alone disease) remain poorly understood. Clinical studies of the ADHD-epilepsy connection are complicated due to its bidirectional nature [4, 5], and thus by difficulties with separating causes from consequences. With this regard, animal models may be useful, as they afford reproducible systems in which either epilepsy or a neurobehavioral disorder of interest represents an unequivocal and an on-demand primary pathology; furthermore epilepsy comorbidities can be examined in the absence of iatrogenic neurobehavioral abnormalities, the latter being attributed to some antiepileptic drugs, such as phenobarbital [6, 7], gabapentin [8, 9], valproate [10, 11] and topiramate [12, 13]. There has been growing evidence that rodent models of acquired chronic epilepsy are characterized not only by spontaneous recurrent seizures, but also produce a spectrum of neurobehavioral impairments, some of which have been validated as experimental equivalents of neurobehavioral comorbidities of epilepsy [14–19].

The present work originates from our findings that rats with chronic epilepsy develop specific behavioral, biochemical and neuroendocrine impairments indicative of depression [20–23]. Further analysis of animals' behavior suggested that some animals exhibited elements of impulsivity, instead of depressive behavior. This led us to employ a specific ADHD-relevant assay [24–26] in order to explore whether these animals indeed develop ADHD-like abnormalities. Furthermore, considering that central noradrenergic dysfunction has been implicated in mechanisms of both ADHD [27–30] and depression [31–34], we explored whether epileptic animals, along with/instead of the already established suppression of serotonin (5-HT) transmission in the raphe nucleus-forebrain ascending pathway [20, 23], also exhibit dysfunction in the ascending norepinephrine (NE) pathway.

2. Methods

2.1. Subjects

The experiments were performed in male Wistar rats (Charles River, Wilmington, MA), fifty days old at the beginning of the study, in accordance with the policies of the National Institutes of Health and regulations of the UCLA Office of Protection of Research Subjects.

2.2. Induction of chronic epilepsy

Animals received intraperitoneal injection of LiCl (128 mg/kg, Sigma, St. Louis, MO), and 24 hours later - subcutaneous injection of pilocarpine HCl (40 mg/kg, Sigma). The resulting status epilepticus (SE) was characterized by continuous secondary generalized clonic and clonic-tonic seizures starting from 10–15 min after pilocarpine injection. One, four and eight hours after seizure onset, rats were injected with diazepam (10 mg/kg) and phenytoin (50 mg/kg) in order to limit neuronal injury and to mitigate subsequent chronic epilepsy [20, 22]. In control animals, pilocarpine was substituted with saline.

Beginning from the fourth week after SE, animals underwent four weeks of continuous video monitoring in order to confirm the presence of chronic epilepsy and to select subjects for further studies. Animals were held individually in their cages with free access to food and water (until the commencement of the ADHD test) and 12 hours light-dark cycle (during the latter, LED light was used as a light source). Video was acquired using

PC33CHR-4G digital cameras connected to DMR41DVD Linux-based computer used for data storage. Video was analyzed off-line for the presence of secondary generalized clinic-tonic seizures, corresponding to stages 4–5 on the Racine scale [35]. Only those animals which showed between 1 and 5 seizures per week were used for behavioral assays. This insured the presence of epilepsy, but at the same time limited seizure frequency to a level that rendered those animals amenable to further behavioral tests [20, 22].

2.3. Forced swimming test (FST)

FST is used as a test for hopelessness/despair (which is a key symptom of depression), whereby the animal's ability to effectively cope with an inescapable stressful situation is quantified [36–38]. The test was conducted at the end of video monitoring. FST consisted of a single five-minute swimming session in a tank filled with water at 22°-25°C [20, 37, 39]. Swimming behavior was videotaped and analyzed offline. Three types of behavior were analyzed (Supplementary data, video). (i) active swimming, representing attempts to escape from the tank: swimming along the walls, climbing on the walls of the tank (effective coping); (ii) immobility: movements' were limited to maintaining head above the water, without attempts to escape (no coping); (iii) non-cued struggle: actively treading water away from the walls, without attempts to escape (ineffective coping). Swimming sessions were videotaped; cumulative duration of immobility and non-cued struggle was calculated by two independent observers. Based on our earlier report, the increase of immobility time in epileptic animals was designated as either moderate, when it did not exceed 100 s (i.e. no more than 30% or total test duration) or severe, when its cumulative duration was 100 s or more [23]. For each parameter, the average duration from the two observations was used. Cumulative duration of active swimming duration was derived by subtracting the sum of immobility and struggling from 300 s (i.e. total test duration).

2.4. Lateralized reaction-time task (LRTT)

LRTT was used to examine animals' impulsivity and attention [24–26]. The test started within one week after the FST. Prior to the inception of testing, *ad libitum* feeding was ended; instead, food was provided in limited amount to the rats once per days. The amount that was fed to each subject was individualized in order to reduce their weights to 80–85% of their initial, *ad libitum* feeding weights and to maintain it at this level through the period of testing. Once testing began, this daily feeding was provided 1–3 hrs after the completion of testing.

Behavioral testing apparatus—Standard extra tall aluminum and Plexiglas operant conditioning chambers with a curved panel fitted with a horizontal array of five nose poke apertures on one side and a photocell-equipped pellet receptacle on the other side (Med Associates, Mt Vernon, VT, USA) were used. The boxes were housed inside of a sound-attenuating cubicle with ambient white noise (85 dB) broadcast to mask external noise; the environment was illuminated with a house light diffuser that was positioned outside of the testing chamber, providing indirect illumination of the testing environment.

Pretraining—All rats were first trained in a single session in which the house light was continuously illuminated and single pellets (45-mg Dustless Precision Pellets; Bio-Serv Inc, Frenchtown NJ) were delivered into an illuminated magazine on a fixed time 30-s schedule over a 45-min period. One day after this session, the rats were trained to make a sustained nose poke at the center aperture in three consecutive daily sessions. On the first day, the session began with illumination of the house light; a variable-duration nose poke of 0.01, 0.2, 0.4, or 0.6 s was required in the illuminated center aperture to trigger a pellet to be dispensed within the head entry magazine on the back wall (the nose poke duration requirements were varied randomly from trial to trial). When the rat successfully responded

for the duration of the hold period, the head entry magazine was illuminated and a pellet was dispensed. After the rat retrieved the pellet, the magazine light was extinguished, and 3-s later, the center aperture was illuminated to signal the initiation of another trial. The session terminated after 60 min passed or the rat earned 100 pellets, whichever occurred first. On the second and third days, the procedure was identical except that the rat was required to sustain 0.01, 0.2, 0.5, or 0.7-s nose pokes or 0.2, 0.5, 0.7, or 1.0-s nose pokes, respectively.

Acquisition of the task—After being trained to make the sustained nose poke, rats began daily testing on the LRTT; in the first four sessions, a target stimulus of fixed duration was presented for all trials in a session (which terminated after 60 min or 128 trials, whichever came first). The task began with the illumination of the house light and the rats retrieving a single pellet from the magazine. The center aperture on the opposite wall was illuminated 3 s later. The rat was then required to make a sustained, variable-duration nose poke (0.2, 0.5, 0.7, or 1.0 s) in the center aperture. After the observing response was completed, the far left or far right aperture was illuminated for a fixed period (30, 5, 2.5, or 1 s). During target presentation, a nose poke response at that aperture resulted in a pellet being delivered at the magazine, and a “correct” choice was scored. A limited hold period also applied on days 3 and 4; a response within 5 s of onset of target illumination was reinforced. Three seconds after the pellet was retrieved, the center aperture was illuminated to signal the onset of another trial. When a rat responded at a location that was not that of the target during target presentation or within the limited hold period, all lights in the box were extinguished, and the rat was given a 3-s “time-out” period in complete darkness; in this case, an “incorrect choice” was scored. In addition, if the rat made no response within target presentation or the limited hold period, the rat received a 3-s “time-out” in darkness and an “omission” was recorded. In both cases, the time-out period was immediately followed by illumination of the house light diffuser and the onset of another trial. An additional contingency was in place to discourage premature responses. If a rat responded to either of the possible target locations before completing the sustained nose poke (and before the target presentation), a 3-s timeout was given (as above), and an “anticipatory response” was scored. The duration of the targets was reduced, on an individualized schedule, only when the rat met a performance criterion of >50 trials completed and >75% accuracy. If rats failed to achieve this criterion at a particular stage in 4 consecutive daily sessions, they were excluded from the study.

Variable target stimulus duration conditions—After the acquisition period, rats were tested in sessions in which the duration of the target stimuli was varied randomly from trial to trial within the session. For the test sessions reported in this study, target stimulus durations ranged between 0.5 and 2.0 s, and a correct response within 3.0 s of target onset was reinforced. The session ended after 60 min or 160 trials, whichever came first. All the other task details were identical to those described above. Dependent measures included: (1) discriminative response accuracy (correct responses/[correct+incorrect responses]), (2) omission rate (percent of total trials), (3) total anticipatory responses, (4) total trials initiated, (5) mean initiation latency/ trial (the average interval between illumination of the center nose poke aperture and the initiation of the observing response), (6) pellet retrieval time (the average interval between pellet delivery and head entry into the magazine), and (7) correct response times (the period between target stimulus onset and a nose poke at the response location). We tested the rats for three consecutive days and pooled the resulting data.

2.5. Fast cyclic voltammetry (FCV)

FCV allows measuring the rate of non-enzymatic oxidation of a phenolic hydroxyl group of a monoamine neurotransmitter to the quinonoid form [40]. FCV in prefrontal cortex (PFC; the latter representing an ADHD- and depression- relevant target of monoaminergic pathways emanating from locus coeruleus [LC] and raphe nucleus [RN] [31, 32, 41]) was

coupled with an electrical stimulation of either LC or of RN to measure the strength noradrenergic and serotonergic tone respectively. The two assays were conducted consecutively in the same animal. Animals were anesthetized with Urethane (1.5 g/kg, i.p.) and positioned into stereotaxis frame (David Kopf Instruments, Tujunga, CA). Nafion-coated carbon fiber electrode (World Precision Instruments, WPI, Sarasota, FL) was placed into PFC (from Bregma: anterior 2.2 mm, left 0.5 mm, ventral 4.5 mm [42]). Reference electrode (Dri-ref, WPI) was placed on the nasal bone. Bipolar twisted stimulating electrode (PlasticsOne, Roanoke, VA) was first placed into LC (from Bregma: posterior −9.7 mm, left 1.2 mm, ventral 7.2 mm [42]) and connected to the DS8000 stimulator via DSI100 isolating unit (WPI). Auxiliary platinum electrode was placed 1 mm from carbon fiber electrode, on the brain surface. The three electrodes were connected to the POT-500 potentiostat (WPI), which in turn was connected to the MP-100 acquisition system (Biopac, Santa Barbara, CA). After finishing data acquisition from the LC-PFC pathway, the stimulating electrode was moved into dorsal raphe nucleus (from Bregma: posterior 7.8 mm, midline, ventral 6.4 mm [42]). The position of carbon fiver electrode remained the same. To detect NE release in the LC-PFC projection, ramp current was applied to the carbon fiber electrode first in the absence of LC stimulation (to detect baseline responses): rest potential 0.4 V scanned to 1.3 V, then to −0.4, at a rate of 400 V/s (Supplementary Figures 1 and 2) [43, 44]. The current was applied 10 times with 100 ms intervals. The procedure was repeated together with electrical stimulation delivered to the stimulating electrode, to detect evoked responses: twenty bipolar square wave, 100 ms, 20 Hz, 5 mA [43, 44]. The procedure was repeated 5 times, every 10 minutes. The specificity of the technique towards NE was confirmed in separate studies (Supplementary Fig. 1).

To measure 5-HT release, ramp current was applied to the carbon fiber electrode first without raphe stimulation, in order to detect baseline responses: rest potential 0.2 V scanned to 1 V, then to −0.1 and then back to 0.2 mV, at a rate of 1000 V/s (Fig. 5B1) [20, 23, 39, 45, 46]. The current was applied 10 times with 100 ms intervals. Then, the procedure was repeated together with electrical stimulus applied to stimulating electrode, to detect evoked responses: five bipolar square wave pulses, 200 ms, 100 Hz, 0.35 mA. The procedure was repeated 10 times, every 5 minutes. The specificity of the procedure to detect 5-HT transmission was previously validated in our lab [20, 23, 39].

For both NE and 5-HT measurements, averages of all waveforms were used for statistical analysis. The amount of transmitter released was calculated by subtracting average evoked response from baseline response. The resulting faradaic currents reflect the amount of a monoamine oxidized/released under the carbon fiber electrode in response to standard stimulus applied to the source area.

2.6. Data analysis

Data were analyzed using Prizm 5 software (GraphPad, San Diego, CA). Statistical tests and sample sizes are indicated in respective portions of the Results and in Figure legends. Parametric tests were used based on the values Gaussian distribution (D'Agostino and Pearson omnibus normality test).

3. Results

3.1. Forced swimming test

The test was performed in 17 epileptic and 8 naïve rats. Consistent with our earlier findings [20–22, 39], post-SE animals showed increased immobility time, thus pointing to the state of despair/hopelessness (Fig. 1A). Further analysis of animals' behavior showed that even though the immobility time varied among post-SE animals (as this has been shown earlier

[23]), the cumulative duration of active swimming remained consistently reduced. Instead, post-SE animals with moderate increase of the immobility time (i.e. those in which immobility time did not exceed 100 s), displayed significant increase of the non-cued struggle (Fig. 1A, B). At the same time, in animals with severe increase of immobility (i.e. over 100 s), non-cued struggle was in normal range (i.e. the one observed in naïve rats, Fig. 1A, B). In our previous report we described post-SE animals as severely and moderately depressed, based on the extent of the increase of the immobility time [23]. In the present study, based on new observations, we modified these definitions by assigning animals to "immobile" (immobility >100, struggle in normal range, n=11) and "struggling" (immobility <100s, increased non-cued struggle, n=6) groups.

3.2 Lateralized reaction-time task

Out of 17 post-SE and 8 naïve animals which entered the study, 13 post-SE and 6 naïve animals met training criteria and were tested under the variable target duration conditions. Post-SE animals showed exacerbated impulsive behavior, evident as significant increase in the number of impulsive responses, as compared with naïve subjects (Fig. 2A). For the purpose of further analyses, post-SE animals were divided into two categories: those in which percent of impulsive responses did not exceed the maximal respective parameter in naïve animals were described as "non-impulsive"; rats in which percent of impulsive responses exceeded the maximal respective parameter in naïve animals, were described as "impulsive" (Fig. 2A, Fig. 3). Along with the increased impulsivity, post-SE animals exhibited diminished attention, which showed statistical significance compared with naive rats at the 0.5 s stimulus duration (Fig. 2B). Further individual analysis showed that only "impulsive" post-SE animals exhibited lower number of correct responses (Fig. 3); therefore, there was a congruency between the increased impulsivity and diminished attention.

3.3. Relationship between behaviors in LRTT and FST

Comparison of animals' behavior in the LRTT and FST revealed that all "non-impulsive" post-SE animals showed significant increase in the immobility time (i.e. corresponded to the "immobile" FST group), while impulsive animals showed both moderate and severe increase in the immobility. Therefore, although in both "non-impulsive" and "impulsive" groups cumulative immobility time was longer than in naïve animals, in "non-impulsive" rats the immobility duration significantly exceeded the one in "impulsive rats" (Fig. 4A). Congruently with this observation, 6 out of 7 "impulsive" post-SE rats showed increased non-cued struggle (i.e. belonged to the "struggling" group), while in all "non-impulsive" animals cumulative duration of non-cued struggle was comparable to that in naïve subjects (Fig. 4B).

3.4. Impairments in noradrenergic and serotonergic transmission in relation to behavioral abnormalities

Post-SE animals showed various patterns of impairments of noradrenergic transmission in the LC-PFC projection and of serotonergic transmission in RN-PFC pathway. These patterns included normal NE release combined with the suppressed 5-HT release; suppression in both 5-HT and NE transmission, and suppressed NE release coupled with preserved 5-HT responses (Fig. 5; Supplementary Figure 2).

In the group of all post-SE rats combined, NE release was significantly suppressed as compared with naïve subjects. However, on the category level, all "impulsive" animals exhibited significantly compromised NE-release, while the parameter remained normal in 5 out of 6 "non-impulsive" animals (Fig. 5 A, B). Consistent with earlier reports [20, 23], there was an overall suppressed 5-HT release from RN into the PFC in post-SE animals

(Fig. 5A). On the category level, all “non-impulsive” rats showed compromised serotonergic transmission ($p<0.05$ vs. Naïve), while “impulsive” animals showed both normal (n=4) and diminished (n=3) 5-HT output ($p>0.05$ vs. Naïve; Fig. 5 A, C).

Congruently with these observations, post-SE animals exhibiting an exacerbated non-cued struggle in the FST, showed diminished NE output in the LC-PFC projection and preserved 5-HT release in the RN-PFC pathway. At the same time, in animals of the “immobile group”, there was a consistent decrease of 5-HT transmission; NE release remained normal in 6 rats, and was diminished in 5 animals (Fig. 6).

Cross-analysis of behavioral and biochemical responses in epileptic rats shows that the suppressed NE release in the LC-PFC pathway is a common attribute of the increased impulsivity in the LRTT and of the increased non-cued struggle in the FST; at the same time, compromised serotonergic transmission in the RN-PFC projection is a hallmark of normal impulsivity coupled the exacerbated immobile behavior (Table 1).

4. Discussion

Approximately one half of pilocarpine rats displayed increased impulsivity and diminished attention in the LRTT. These impairments were consistently associated with the suppressed noradrenergic tone in the LC-PFC pathway. Furthermore, there existed a dichotomy in chronic sequelae of SE in that the other half of animals showed significant increase of the immobility time in the FST in association with the serotonergic deficit in the RN-PFC pathway [20, 23]. The diversity of behavioral outcomes of SE raises a question: why does a similar epileptogenic insult produce different behavioral abnormalities in different animals? Gender and age of the insult can be rejected based on the study design. Genetic predisposition is not impossible despite relative genetic homogeneity of the animals by the virtue of the strain. Indeed the development of rat strains which model major depression [36], ADHD [47], absence epilepsy [48] and high propensity to kindling epilepsy [16] has been based on inbreeding of outlier subjects presenting with a phenotype of interest. Therefore, some Wistar rats may have genetic propensity to depression and others- to ADHD, both of which are idle, but are triggered by the epileptic process. However, more simple explanations would lie in the severity and the pattern of epilepsy, and of neuronal injury produced by SE.

The severity of chronic epilepsy significantly varies in the pilocarpine model, both among animals and even for the same animal during the course of the disease [49–51]. Due to logistical issues (particularly incompatibility between the environment for the ADHD experiments and the one for seizure monitoring), we were not able to assess seizure frequency throughout the experiments; nor could we examine sub-clinical seizures using EEG. However, there was no association between the frequency of stage 4–5 seizures during the pre-selection period and patterns of behavioral abnormalities (Supplementary Table 1). Furthermore, the congruency between the non-cued struggle in the FST (the latter being performed immediately after seizure monitoring) and the increased impulsivity in the LRTT on the one hand, and lack of association between the non-cued struggle and spontaneous seizure frequency on the other hand, corroborate the lack of connection between seizure frequency and ADHD-like behavior. With regard to clinical relevance, presently there is no consensus as to the correlation between the frequency of seizures or the degree of seizure control and the severity of comorbid ADHD in epilepsy patients [1]. In our system, impulsive and attention abnormalities were clearly triggered by SE; however, they tended to evolve notwithstanding the severity of epilepsy. A more definitive answer may be obtained through chronic suppression of spontaneous seizures by antiepileptic medications and examining whether ADHD-like impairments would persist. Alternatively, employing a

model in which chronic epileptic state is created in the absence of spontaneous seizures (such as kindling), could be instructive. In fact, inbred rats with an inherently increased propensity to kindling showed inferred elements of diminished attention and impulsivity in the Morris water maze and escape from restraint paradigms [52, 53], as compared with normal animals; however, since no ADHD-specific tasks were employed, no unequivocal conclusions can be drawn from these studies. On a related note, depressive behavior in the pilocarpine model does not depend on the frequency of spontaneous seizures [20], and rats that have undergone hippocampal kindling exhibit depression-like impairments despite the absence of spontaneous seizures [54].

Besides the severity, the pattern of seizures may be important. ADHD is more commonly associated with frontal lobe epilepsy and absence epilepsy, than with temporal lobe epilepsy (TLE) [2, 3]. While pilocarpine model is primarily regarded as a model of TLE [55], other seizure types are also observed. Neuroprotection in limbic areas afforded by acute treatment of pilocarpine SE by carisbamate resulted in the displacement of secondary generalized complex partial seizures by absence-like seizures [56]. SE induced by pilocarpine in neonatal rats (the latter developing significantly milder injury in limbic structures than adults [49, 57, 58]), resulted in absence-like seizures during the adulthood [59]. It is therefore possible that allowing SE to resolve without diazepam+phenytoin treatment would prevent the occurrence of ADHD-like abnormalities. However, such studies would likely be complicated, as the resulting high frequency of generalized seizures would interfere with the animals' training and performance in operant tasks like the one used here; in fact, we introduced the diazepam+phenytoin regimen with the specific purpose of making epileptic animals amenable to the FST [20, 21]. There are no conclusive reports on the presence of frontal lobe seizures in the pilocarpine model. However, several studies have shown the up-regulation of NMDA and down-regulation of GABA-A receptors [60], increased lipid peroxidation [60–62], increased theta rhythm power [63] in frontal/prefrontal cortex, all pointing to chronic perturbations in excitability occurring in this area.

Since major depression is commonly encountered among TLE patients [64–66], the dominating TLE pattern following pilocarpine SE appears to be a reasonable cause of depression-like abnormalities. Our earlier studies outlined events leading to depression in this model, starting from the upregulation of interleukin-1β in the hippocampus, subsequent hyperactivity of the hypothalamo-pituitary-adrenocortical axis, the upregulation of raphe 5-HT1A autoreceptors and ultimately inadequate 5-HT release from RN [20–23].

Neuronal injury following pilocarpine SE spreads beyond limbic structures and the severity of the injury in different brain sites significantly varies [49, 57, 58, 67–69] (likely due to unpredictable, random recruitment of different neuronal circuits following systemic pilocarpine administration), thus possibly translating in different behavioral outcomes. Neurochemical assays in our study rendered brains not usable for histological examination; the latter would require a thorough stereological assessment of neuronal cell loss. On a preliminary note however, the protocol that we employ does not produce severe chronic neurodegeneration in both hippocampus and PFC, although acute/sub-acute injury (and thus plausible chronic impairments in neuronal plasticity) takes place in both areas (Supplementary Fig. 3). To summarize, the contribution of genetic predisposition, severity and pattern of spontaneous seizures and of neuronal injury into the observed behavioral outcomes of SE cannot be ruled out. Most conceivably, the type of behavioral abnormalities depends on a stochastic recruitment of relevant neuronal pathways and their chronic maladaptive perturbations. Among the discussed variables, the type of spontaneous seizures, particularly frontal lobe and/or absence seizures vs. complex partial secondary generalized seizures appears to be probable determinants of ADHD and depression respectively;

however, this has to be corroborated in the longitudinal EEG and video monitoring experiments.

At the same time, pilocarpine model still offers opportunities for examining mechanisms of comorbidity between epilepsy and either ADHD or depression. Indeed, we found that ADHD-like and depression-like impairments were accompanied by specific perturbations in central monoaminergic transmission.

It has been established earlier that animals with severely exacerbated immobility in the FST showed suppression of 5-HT release from the RN into the forebrain [23]. Here, we also found that there was no consistent association between the noradrenergic dysfunction and the severity of depressive behavior. At the same time, animals with the increased impulsivity/diminished attention exhibited consistent noradrenergic hypo-function, while serotonergic transmission did not correlate with ADHD-like abnormalities (Table 1). The involvement of serotonergic transmission in mechanisms of major depression is well established. Dysfunction of noradrenergic transmission has also been suggested; this may involve both hyper- and hypo-function of LC outputs into the neocortex, hippocampus and ventral tegmental area [31, 32, 70]. However, our experiments show that noradrenergic dysfunction is not necessary for the development of depressive abnormalities at least in the pilocarpine model.

Mechanisms of ADHD remain poorly understood. A dominating theory implicates dopaminergic dysfunction, and psychostimulants are most commonly used for the ADHD treatment [71]. Perturbations in noradrenergic transmission, specifically in the LC-PFC pathway, has also been suggested, and a norepinephrine reuptake inhibitor atomoxetine or alpha-2 noradrenergic receptor agonist guanfacine are the only non-psychostimulant drugs approved for the treatment of ADHD [27, 71]. However, the direction in which noradrenergic dysfunction occurs, remains subject of debates. Both hyper- and hypo-activity of LC-PFC projection have been shown [27, 72], but our studies are congruent with the latter findings. Causes of maladaptive changes in noradrenergic transmission remain elusive as well. The excitatory input from PFC into LC represents a major modulator of the activity of LC neurons and subsequently determines the tone of the LC-PFC noradrenergic pathway [73, 74]. Here again, some studies show that PFC neurons activate LC noradrenergic cells [75], while other studies show the opposite [76]. The discussed perturbations in PFC in the pilocarpine model, as well as at least transient neuronal injury in this area suggest that chronic maladaptive changes in PFC following SE may modulate the activity of LC neurons.

Another question of interest is whether ADHD-like and depressive abnormalities may occur in the same animal. This is particularly relevant given a well-established comorbidity between major depression and ADHD [77, 78]. Although in 5 out of 17 rats both serotonergic and noradrenergic tones were suppressed, 5-HT deficit was not necessarily associated with ADHD, and NE deficit- with depressive behavior. Further, only 2 out of 7 animals with the increased impulsivity showed severe increase in the immobility time. Therefore it appears that at least “severe depression” is not compatible with ADHD-like behavior in the pilocarpine model. Furthermore, there has been clear dichotomy between the immobility and the non-cued struggle in the FST, and the latter behavior strongly paralleled impulsivity in the LRTT.

At the same time, all animals showed at least moderate increase of the immobility time in the FST. We previously hypothesized that severe and moderate increase of immobility in epileptic rats may have different underlying mechanisms [23]. The interpretation of moderate increase in the immobility is complicated. It may reflect “moderate depression”, thus suggesting that these animals develop “triple morbidity”, that is epilepsy-depression-

ADHD. Along these lines, it is also possible that at least some of "severely depressed" animals could be presenting with ADHD-like abnormalities; however the latter could not be revealed in the operant test we used here due to the lack of motivation associated with depression (the test involves positive reinforcement of behavior using a palatable food reward). Indeed, depression is commonly known to mask ADHD symptoms, thus complicating the diagnosis of the latter [77, 78]. Alternatively, moderate increase in the immobility may merely reflect the increased fatigue following motor hyperactivity during struggling episodes.

These considerations emphasize the need for cautious interpretation of animals' behavior particularly in connection with epilepsy. For example, decreased level of anxiety in the elevated plus maze test (EPMT) was reported in animals with pilocarpine epilepsy [79]. Our studies (Supplementary Fig. 4) revealed high degree of correlation between the decreased anxiety in the EPMT and non-cued struggle (read: increased impulsivity) in the FST. It is thus possible that behavior displayed by epileptic rats in the EPMT represents a non-specific manifestation of impulsivity rather than a true decrease in anxiety.

5. Conclusions

Rats with chronic epilepsy exhibit divergent interictal behavioral abnormalities, which suggest the comorbidity between epilepsy and either ADHD or depression. The observed behavioral impairments correlate with specific perturbations in central monoaminergic pathways. Thus, pilocarpine model provides a system which can be used to examine mechanisms underlying these common comorbidities of epilepsy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

5-HT	serotonin
ADHD	attention deficit and hyperactivity disorder
EPMT	elevated plus maze test
FCV	fast cyclic voltammetry
FST	forced swimming test
LC	locus coeruleus
LRTT	lateralized reaction time task
NE	norepinephrine
PFC	prefrontal cortex
RN	raphe nucleus
SE	status epilepticus
TLE	temporal lobe epilepsy

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Highlights

- Chronic epileptic rats exhibit either increased impulsivity, or depressive behavior
- Hyper-impulsivity correlates with the suppressed central noradrenergic tone
- Depressive behavior correlates with the suppressed central serotonergic tone
- ADHD-like and depressive impairments are mutually exclusive

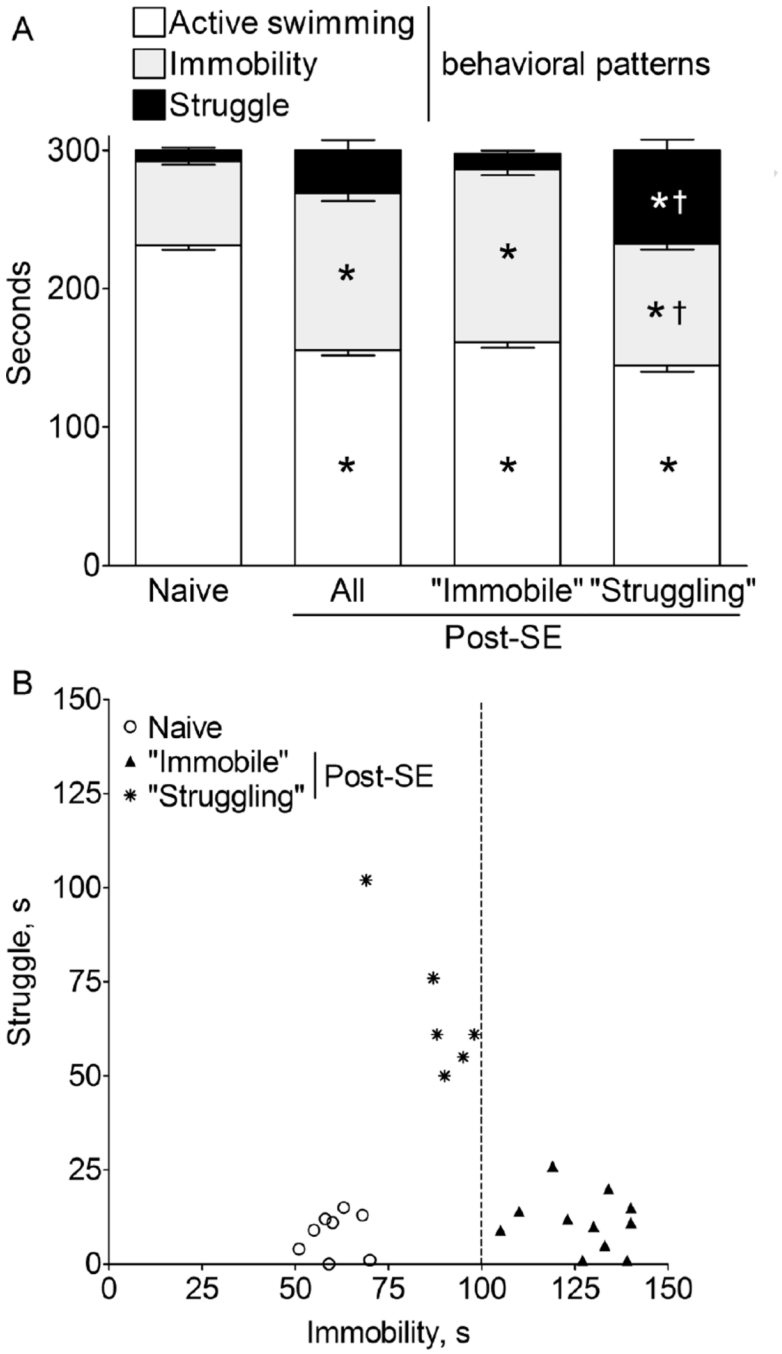

Figure 1. Forced swimming test

Post-SE animals showed increase in cumulative duration of immobility time and increased non-cued struggling behavior. A. Group data are presented as Mean±SEM. *- $p<0.5$ vs. Naïve for each respective parameter (i.e. swimming, immobility and struggle); †- $p<0.05$ "Struggling" vs. "Immobile" groups. All vs. Naïve- t-test. "Immobile" vs. "Struggling" vs. Naïve- One Way ANOVA + Bonferroni multiple comparison test (active swimming $F(2,22)=126.2$, $p<0.0001$; immobility $F(2,23)=78.13$, $p<0.0001$; struggling $F(2,22)=63.84$, $p<0.05$). B. Plot of individual data showing the dichotomy between the immobility and struggle among post-SE animals.

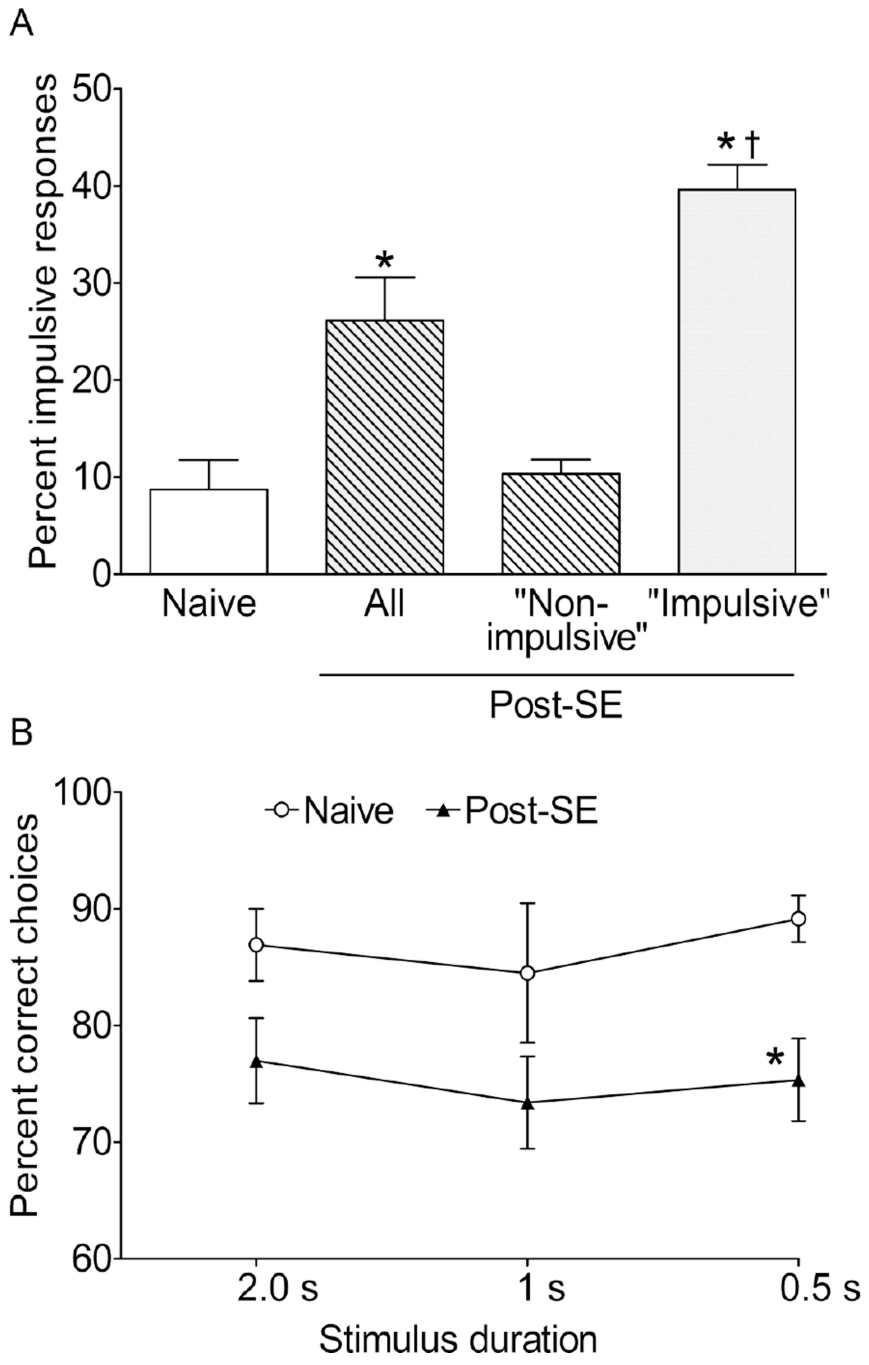

Figure 2. Lateralized reaction time task

Post-SE animals showed increased impulsivity and diminished attention. Data are presented as Mean±SEM. A. Impulsivity *-p<0.05 vs. Naïve; †-p<0.05 “Non-impulsive” vs. “Impulsive” groups. All vs. Naïve-t-test. “Non-impulsive” vs. “Impulsive” vs. Naïve- One Way ANOVA + Bonferroni multiple comparison test ($F(2,16)=53.58$, $p<0.0001$). B. Attention. There was a consistent trend in the reduction of correct choices between Post-SE and naïve animals; however statistical significance was reached only at 0.2 s. *-p<0.05 vs. Naïve (t-test).

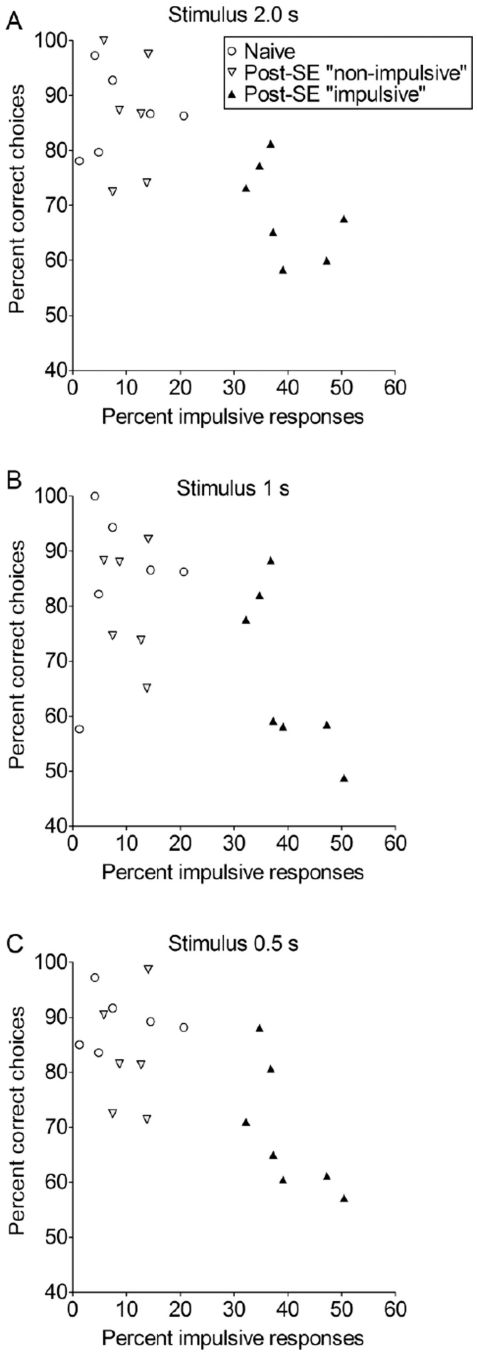

Figure 3. Individual plots of impulsivity vs. attention in the Lateralized reaction time task

A-C show percent of correct choices in response to stimuli of various durations. Only animals with the exacerbated impulsivity showed diminished attention. At 2.0 s and 0.5 s stimulus duration, “impulsive” post-SE animals showed statistically significant decrease in percent of correct choices compared with both naïve and “non-impulsive” rats (2.0s $F(2,16)=7.962$, $p<0.05$; 0.5 s $F(2,16)=7.512$, $p<0.05$).

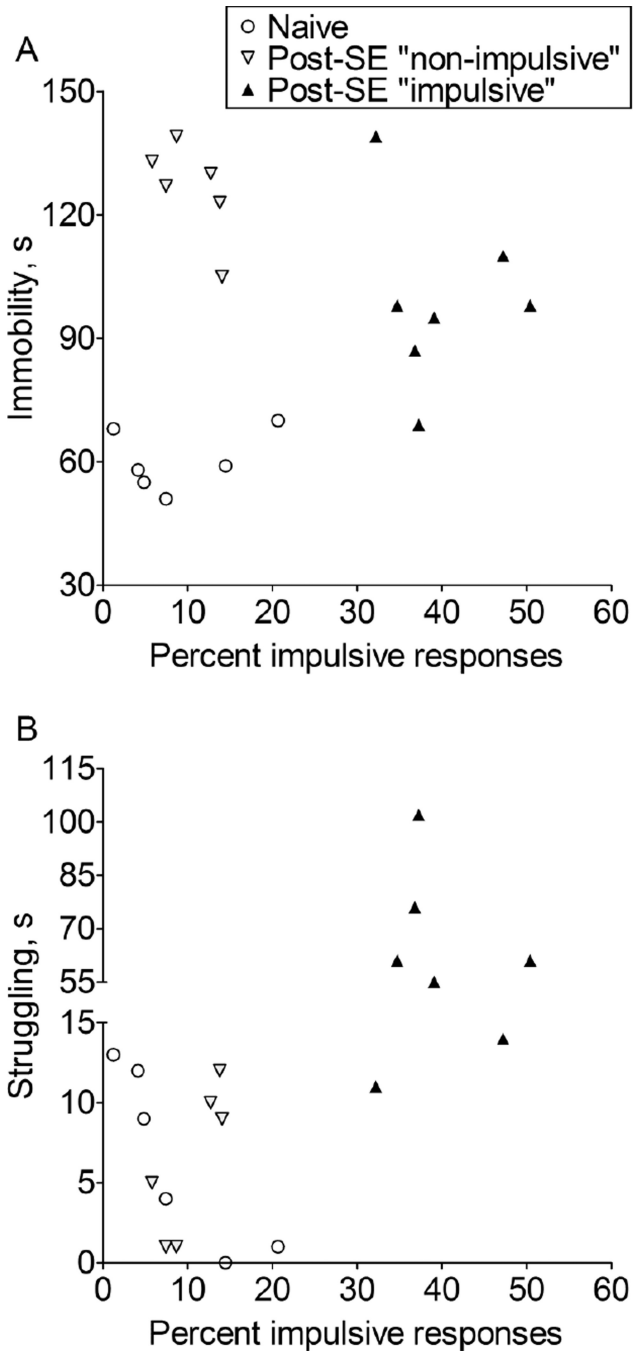

Figure 4. Individual plots of impulsivity in the lateralized reaction time task vs. behaviors in forced swimming test

A. Impulsivity (LRTT) vs. immobility (FST). All post-SE animals showed increased immobility time ($p<0.05$ for "non-impulsive" vs. naïve and "impulsive vs. naïve); however the increased in the immobility was more pronounced in "non-impulsive" rats than in animals of "impulsive" group ($p<0.05$). One-Way ANOVA+Bonferroni multiple comparisons test ($F(2,16)=28.31$, $p<0.0001$). B. Impulsivity (LRTT) vs. non-cued struggle (FST).In "non-impulsive" rats, cumulative duration of non-cued struggle was in normal range ($p>0.05$ vs. naïve), while "impulsive" rats showed significant increase in the non-cued

struggle vs. both naïve and “non-impulsive” animals ($p<0.05$; One way ANOVA + Bonferroni test for multiple comparisons, $F(2,16)=12.27$, $p<0.005$).

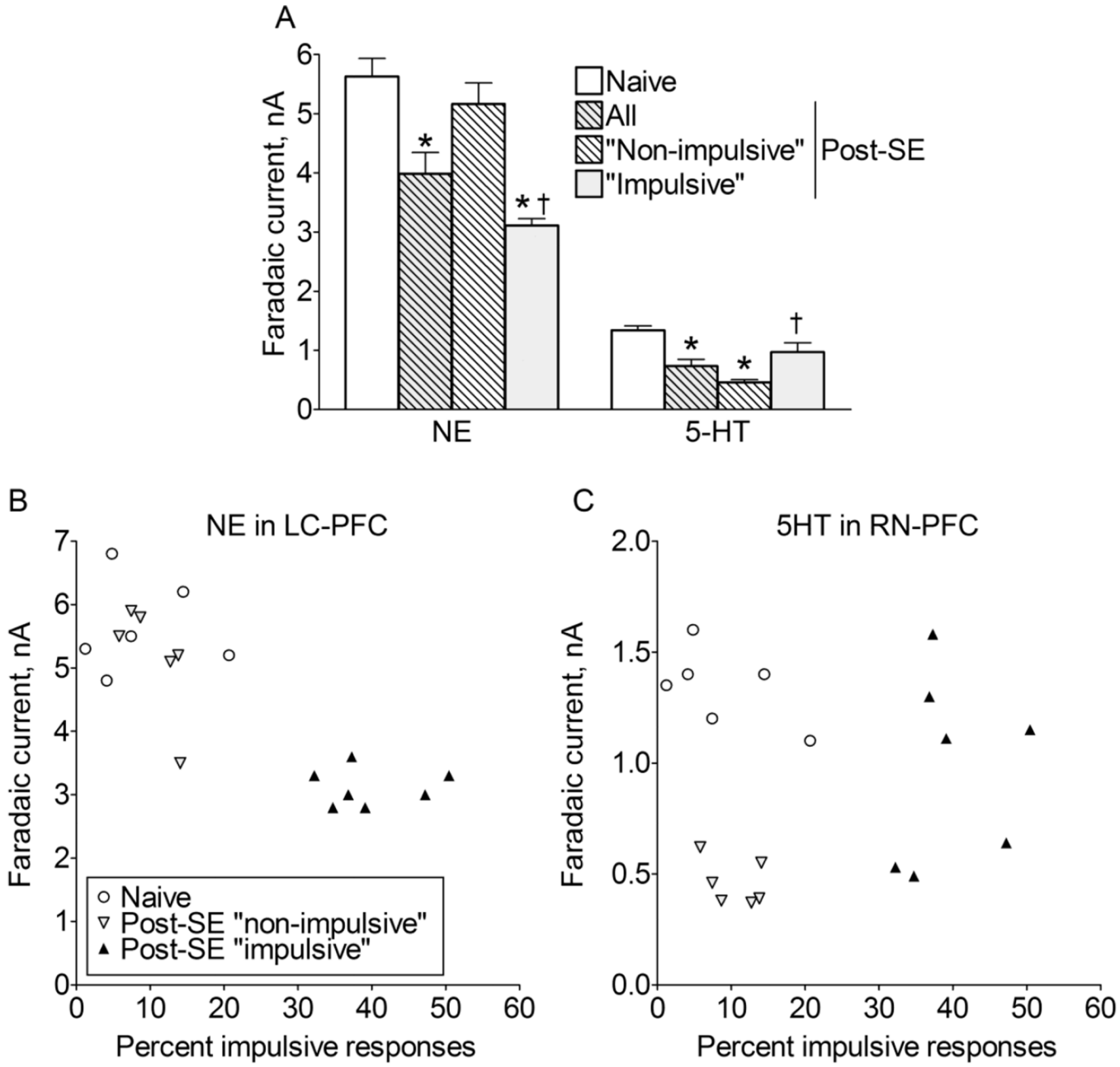

Fig. 5. Noradrenergic and serotonergic transmission with the reference to impairments in the lateralized reaction time task

A. Group data are presented as Mean±SEM. There was an overall decrease in both NE and 5-HT release in all post-SE animals as compared with controls (t-test). “Non-impulsive” animals showed suppressed 5-HT release and preserved NE release; “impulsive” animals showed preserved 5-HT release and suppressed NE release (One-Way ANOVA + Bonferroni multiple comparison test; NE $F(2,16)=26.93$, $p<0.05$; 5-HT $F(2,16)=14.76$, $p<0.05$). *- $p<0.05$ vs. Naïve; †- $p<0.05$”impulsive” vs. “non-impulsive” group. B and C: Individual plots. Individual impulsivity data (LRTT) are plotted against NE release (FCV, B) and against 5-HT release (FCV, C). “Non-impulsive” animals showed consistent suppression of 5-HT release, while “impulsive” rats showed consistently diminished NE release.

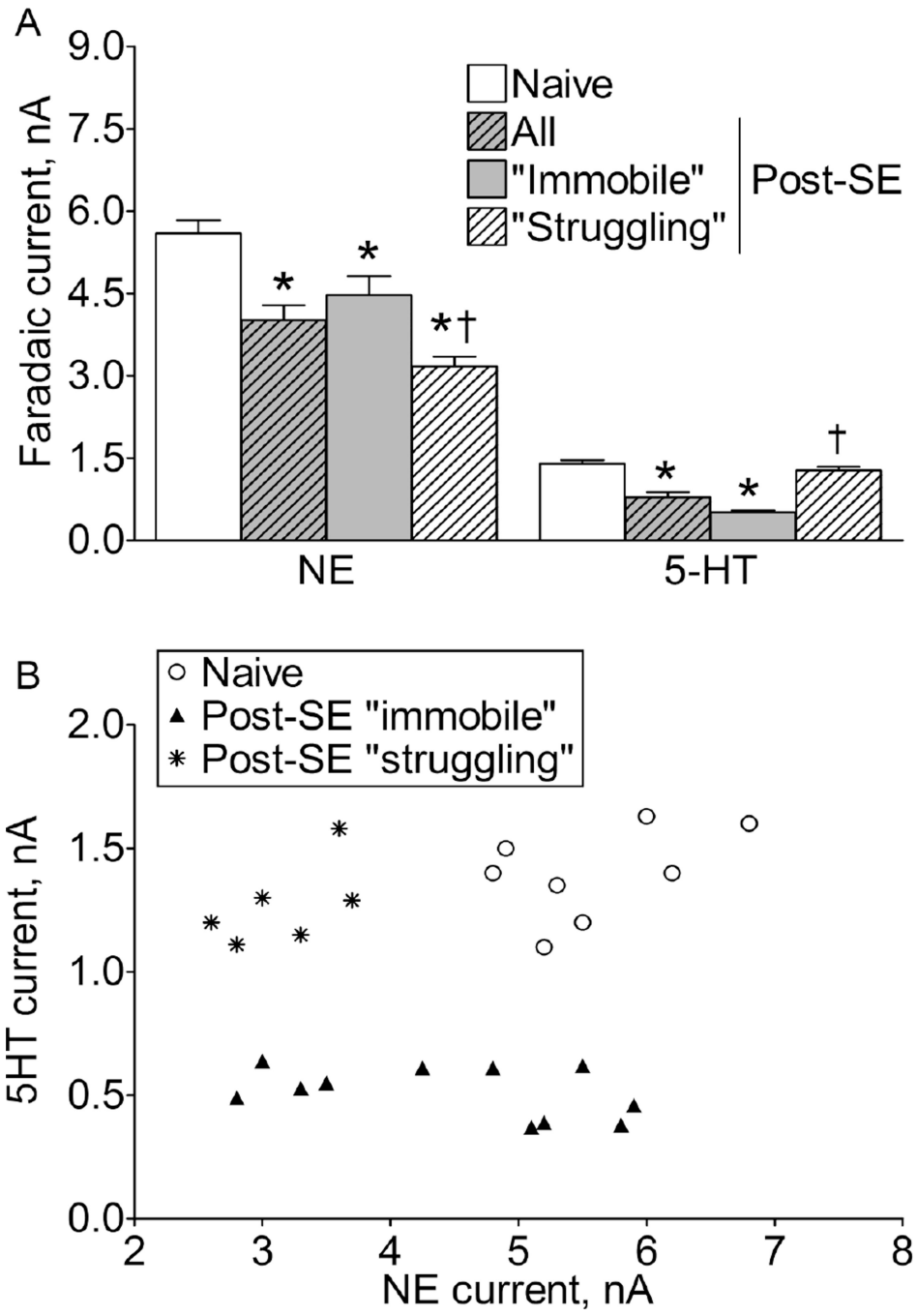

Fig. 6. Noradrenergic and serotonergic transmission with the reference to impairments in the forced swimming test

A. Group data are shown as Mean±SEM. NE release was significantly suppressed in all post-SE animals However, rats of the “struggling” group showed more pronounced suppression than “immobile” animals. There was an overall suppression of 5-HT release in post-SE rats; however, on the sub-group level, only “immobile” animals showed diminished 5-HT responses. *-$p<0.05$ vs. naïve; †- $p<0.05$, “struggling” vs. “immobile” group. (All vs. Naïve- t-test; “immobile” vs. “struggling” vs. naïve- One-way ANOVA+Bonferroni multiple comparison test; NE $F(2,22)=12.62$, $p<0.01$; 5-HT $F(2,22)=98.01$, $p<0.0001$). B. Individual plots whereby noradrenergic responses are plotted against serotonergic responses.

Note the suppression of serotonergic responses in “immobile”, and noradrenergic- in “struggling” animals.

Table 1

Summary of behavioral and biochemical impairments in chronic epileptic rats.

Behavior		Monoaminergic tone (FCV)	
Test	Outcome	NE in LC-PFC	5-HT in RN-PFC
LRTT	Increased impulsivity	Suppressed	Normal or suppressed
	Normal impulsivity	Normal	Suppressed
FST	Increased immobility time, normal non-cued struggle time	Suppressed or normal	Suppressed
	Normal immobility time, increased non-cued struggle time	Suppressed	Normal