Glucocorticoid Induces Incoordination between Glutamatergic and GABAergic Neurons in the Amygdala

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Abstract

Background
Stressful life leads to mood disorders. Chronic mild stress is presumably major etiology for depression, and acute severe stress leads to anxiety. These stressful situations may impair hypothalamus-pituitary-adrenal axis and in turn induce synapse dysfunction. However, it remains elusive how the stress hormones mess up subcellular compartments and interactions between excitatory and inhibitory neurons, which we have investigated in mouse amygdala, a structure related to emotional states.

Methods and Results
Dexamethasone was chronically given by intraperitoneal injection once a day for one week or was acutely washed into the brain slices. The neuronal spikes and synaptic transmission were recorded by whole-cell patching in amygdala neurons of brain slices. The chronic or acute administration of dexamethasone downregulates glutamate release as well as upregulates GABA release and GABAergic neuron spiking. The chronic administration of dexamethasone also enhances the responsiveness of GABA receptors.

Conclusion
The upregulation of GABAergic neurons and the downregulation of glutamatergic neurons by glucocorticoid impair their balance in the amygdala, which leads to emotional disorders during stress.

Introduction
Major depression and anxiety are common psychiatric disorders, in which the dysfunctions of the neurons and synapses in the limbic system, such as amygdala, nucleus accumbens and
prefrontal cortex, are presumably involved [1–14]. In terms of etiology for these emotional disorders, the physical and psychological stresses to the genetically susceptible individuals impair the functions of hypothalamus-pituitary-adrenal axis [15–19], and induce the neuron atrophy of the limbic system. To the influence of stress hormones on the functions of the synapses and neurons, previous studies indicate that glucocorticoid regulates the function of GABA_A receptors [20–22] and diminishes the density of GABAergic neurons in prenatal period [23]. Chronic stress deteriorates the reversal potentials and densities of GABA_A receptor-channels [24–26] and the strength of GABA synaptic transmission [27–30]. How stress hormones influence the subcellular compartments of the neurons as well as the interactions between excitatory and inhibitory neurons, i.e., cell-specific pathology, remains to be studied [31], since their coordination is critical for the neuronal encoding to manage well-organized cognitions [32, 33].

The amygdala, an important structure in the limbic system, is involved in the emotional processes including high and low mood states [34–39]. It plays a physiological role in fear memory [40–44]. Its dysfunction is associated to anxiety [5, 6, 45, 46] and major depressive disorder [47, 48]. Glucocorticoid receptors are localized in amygdala neurons [49]. As physiological interactions and balances between glutamatergic and GABAergic neurons are critical for maintaining normal brain functions [50], how glucocorticoids regulate these neurons and their interactions remains to be investigated in the amygdala [51].

In the present study, we aim to examine how glucocorticoids affect the subcellular compartments and interactions between glutamatergic and GABAergic neurons in the amygdala, which may be involved in the pathological processes of major depression and anxiety. Glucocorticoid influence was examined by acutely washing dexamethasone to the brain slices including amygdala or by chronically intraperitoneally injecting dexamethasone in the mice. GABAergic and glutamatergic neurons in these mice are genetically labeled by green and yellow fluorescent proteins, respectively, for cellular identity. Their excitability and synaptic transmission were measured by whole-cell recording at these neurons in the brain slices.

Results

Dexamethasone upregulates GABA release and GABA_A receptor responsiveness in the amygdala

The effect of dexamethasone, a synthetic glucocorticoid, on the action of the inhibitory neurons to the excitatory neurons was studied by recording sIPSCs on glutamatergic neurons in the mouse amygdala, in which the mice were treated by the intraperitoneal injections of dexamethasone (DEX) once a day (40 mg/kg) for a week. This chronic application of DEX appears to increase sIPSC frequency and amplitude (Fig 1A). Fig 1B shows cumulative probability versus sIPSC amplitude, in which sIPSC amplitudes at P_{0.67} are 10.21±1.09 pA in DEX injection (n = 11 cells from 8 mice) and 7.15±0.4 pA in saline injection (n = 13 cells from 6 mice, p<0.05). Fig 1C illustrates cumulative probability versus inter-sIPSC intervals, in which inter-sIPSC intervals at P_{0.67} are 1337±216 ms in DEX injection (n = 11 cells from 8 mice) and 2741±511 ms in saline injection (n = 13 from 6 mice, p<0.05). The chronic application of dexamethasone increases GABA release from inhibitory neurons and GABA_A receptor responses on excitatory neurons in the amygdala.

To examine the acute effect of dexamethasone, we washed DEX (25 μM) onto the brain slices in control mice. This acute application of DEX appears to increase sIPSC frequency (Fig 2A). Fig 2B shows cumulative probability versus sIPSC amplitudes, in which sIPSC amplitudes at P_{0.67} are 4.99±0.62 pA in the control and 5.0±0.56 pA after washing-on DEX (n = 10 cells from 8 mice). Fig 2C illustrates cumulative probability versus inter-sIPSC intervals, in which
inter-sEPSC intervals at $P_{0.67}$ are $2689 \pm 599$ ms in the control and $1128 \pm 248$ ms after washing on DEX (n = 10 cells from 8 mice, $p < 0.05$). The acute application of dexamethasone increases GABA release from inhibitory neurons in the amygdala. We studied whether DEX-induced increase in GABA release was due to an increased excitability in these inhibitory neurons, in which sequential spikes were induced by depolarization pulses (Methods). The chronic application of DEX by its intraperitoneal injection looks to enhance spike frequency (Fig 3A). Fig 3B illustrates that the intervals of spikes 1~2, 2~3, 3~4 and 4~5 are $17.48 \pm 1.73$, $21.85 \pm 2.11$, $24.5 \pm 2.63$ and $26.76 \pm 3.68$ ms in DEX injection (n = 11 cells from 6 mice) and $24.24 \pm 2.38$, $26.71 \pm 2.43$, $33.48 \pm 2.28$ and $43.06 \pm 3.57$ ms in saline injection (n = 11 cells from 6 mice, one asterisk, $p < 0.05$ and two asterisks, $p < 0.01$). Moreover, the acute application by washing DEX onto the brain slices appears to raise spike frequency (Fig 3C). Fig 3D illustrates that the intervals for spikes 1~2, 2~3, 3~4 and 4~5 are $39.17 \pm 4.39$, $39.92 \pm 4.1$, $43.92 \pm 3.2$ and $44.1 \pm 3.37$ ms in control and $31.52 \pm 2.5$, $32.72 \pm 2.29$, $33.85 \pm 2.3$ and $35.53 \pm 2.45$ ms after washing on DEX (n = 13 cells from 10 mice, two asterisks, $p < 0.01$ and three asterisks, $p < 0.001$). Thus, dexamethasone upregulates spike capability in the GABAergic neurons, which may lead to the increased GABA release in the amygdala.

Dexamethasone upregulates the excitability of GABAergic neurons in the amygdala

We studied whether DEX-induced increase in GABA release was due to an increased excitability in these inhibitory neurons, in which sequential spikes were induced by depolarization pulses (Methods). The chronic application of DEX by its intraperitoneal injection looks to enhance spike frequency (Fig 3A). Fig 3B illustrates that the intervals of spikes 1~2, 2~3, 3~4 and 4~5 are $17.48 \pm 1.73$, $21.85 \pm 2.11$, $24.5 \pm 2.63$ and $26.76 \pm 3.68$ ms in DEX injection (n = 11 cells from 6 mice) and $24.24 \pm 2.38$, $26.71 \pm 2.43$, $33.48 \pm 2.28$ and $43.06 \pm 3.57$ ms in saline injection (n = 11 cells from 6 mice, one asterisk, $p < 0.05$ and two asterisks, $p < 0.01$). Moreover, the acute application by washing DEX onto the brain slices appears to raise spike frequency (Fig 3C). Fig 3D illustrates that the intervals for spikes 1~2, 2~3, 3~4 and 4~5 are $39.17 \pm 4.39$, $39.92 \pm 4.1$, $43.92 \pm 3.2$ and $44.1 \pm 3.37$ ms in control and $31.52 \pm 2.5$, $32.72 \pm 2.29$, $33.85 \pm 2.3$ and $35.53 \pm 2.45$ ms after washing on DEX (n = 13 cells from 10 mice, two asterisks, $p < 0.01$ and three asterisks, $p < 0.001$). Thus, dexamethasone upregulates spike capability in the GABAergic neurons, which may lead to the increased GABA release in the amygdala.

Dexamethasone downregulates glutamate release in the amygdala

The effect of dexamethasone on the action of the excitatory neurons to the inhibitory neurons was studied by recording sEPSCs on the GABAergic neurons in the mouse amygdala, in which
the mice were treated by intraperitoneal injections of DEX once a day (40 mg/kg) for one week. This chronic application of DEX appears to reduce sEPSC frequency (Fig 4A). Fig 4B demonstrates cumulative probability versus sEPSC amplitudes, in which sEPSC amplitudes at $P_{0.67}$ are $8.53\pm0.79$ pA in DEX injection ($n = 19$ cells from 6 mice) and $8.58\pm0.67$ pA in saline injection ($n = 12$ cells from 6 mice, $p = 0.9$). Fig 4C illustrates cumulative probability versus inter-sEPSC intervals, in which inter-sEPSC intervals at $P_{0.67}$ are $756\pm94$ ms in DEX injection ($n = 19$ cells from 6 mice) and $428\pm101$ ms in saline injection ($n = 12$ cells from 6 mice, $p < 0.05$). Thus, the chronic application of dexamethasone downregulates glutamate release from excitatory neurons in the amygdala.

To examine this result, we washed DEX (25 μM) onto the brain slices in control mice. This acute application of DEX appears to decrease sEPSC frequency (Fig 5A). Fig 5B illustrates cumulative probability versus sEPSC amplitudes, in which sEPSC amplitudes at $P_{0.67}$ are $10.1\pm1.1$ pA in the control and $10.42\pm0.93$ pA after washing DEX ($n = 14$ cells from 10 mice, $p = 0.8$). Fig 5C shows cumulative probability versus inter-sEPSC interval, in which inter-sEPSC intervals at $P_{0.67}$ are $697\pm105$ ms in control and $1242\pm173$ ms after washing DEX ($n = 14$ cells from 10 mice, $p < 0.001$). Thus, the acute application of dexamethasone decreases glutamate release from the excitatory neurons in the amygdala.
Dexamethasone does not affect the excitability of glutamatergic neurons in the amygdala

In the meantime, we examined whether DEX-induced downregulation in glutamate release is due to the decreased excitability in these glutamatergic neurons. As showed in Fig 6, interspike intervals are not changed by either chronic or acute application of DEX. Therefore, dexamethasone downregulates the subcellular compartment of glutamate release in the excitatory neurons from the amygdala.

Discussion

To the roles of glucocorticoid in regulating interactions between excitatory and inhibitory neurons, our studies demonstrate that dexamethasone upregulates GABA release from inhibitory neurons, GABA<sub>A</sub> receptor responses on excitatory neurons and GABAergic neuron excitability (Figs 1–3). On the other hand, dexamethasone downregulates glutamate release from excitatory neurons (Figs 4 and 5). Taken our data together, we suggest that glucocorticoids lead to the upregulated action of the inhibitory neurons onto the excitatory neurons, as well as the downregulated action of the excitatory neurons onto the inhibitory neurons. We also observed that DEX-induced upregulation of GABA release is not due to the decreased excitability in the inhibitory neurons (Figs 1A and 2A). Therefore, we conclude that dexamethasone regulates neuronal interactions in the amygdala by modulating both excitatory and inhibitory neuron functions.
neurons. The imbalanced interaction between the excitatory and inhibitory neurons toward inhibitory state in neural networks of the amygdala (Fig 7) may be involved in emotional disorders induced by stress hormones.

Previous studies demonstrate that the chronic application of corticosterone impairs hippocampal neurogenesis leading to mood disorders [52, 53] and the acute application of dexamethasone facilitates GABAergic synaptic transmission in the hippocampus [21]. These studies have not compared the acute and chronic effects of glucocorticoids on neuronal activities as well as not examined the influences of glucocorticoids on neuronal activities in the amygdala. In order to address these issues, we have investigated the acute and chronic roles of glucocorticoids in regulating the interactions between excitatory and inhibitory neurons in the amygdala. Our studies suggest that the acute activation of glucocorticoid receptors targets onto the mechanisms relevant to the releases of GABA and glutamate in the presynaptic compartments; however, in addition to the releases of GABA and glutamate, the chronic activation of glucocorticoid receptors acts onto postsynaptic GABA_A receptors (Figs 1 and 2, 4 and 5). The mechanisms underlying these differences are being examined. In terms of the pathological impacts about the differential roles of acute and chronic glucocorticoid receptor activations in the neuron coordination, our explanation is given below. Acute severe stress usually leads to anxiety-related emotion and behaviors, such as posttraumatic stress disorder, while chronic

Fig 4. The chronic application of dexamethasone decreases glutamate release from excitatory neurons and increases glutamate receptor responses in the inhibitory neurons of the amygdala. DEX was used by intraperitoneal injections (40 mg/kg) per day for a week. sEPSCs were recorded in glutamatergic neurons of the brain slices including the amygdala. A) shows sEPSCs in DEX injection (right panel) and saline injection (left). The calibration bars are 6 pA versus 2 seconds (top traces) and 100 ms (bottom traces). B) shows cumulative probability vs. sEPSC amplitudes in DEX injection (red symbols) and saline injection (blue). C) shows cumulative probability versus inter-sEPSC intervals in DEX injection (red symbols) and saline injection (blue).

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mild stress with lack of rewards cause major depressive disorder. Our studies about the differential effects of acute and chronic dexamethasone application on GABA and glutamate release and receptor responses may help to explain the consequences that chronic mild stress causes major depression and acute severe stress leads to anxiety-related disorders.

Our study first shows the different effects of glucocorticoid receptor activation on excitatory and inhibitory neurons. Dexamethasone upregulates spiking ability in GABAergic neurons (Fig 3), but not in glutamatergic neurons (Fig 6). Dexamethasone increases GABA release (Figs 1 and 2) and lowers glutamate release (Figs 4 and 5). Thus, the activation of glucocorticoid receptors may shift the balances between excitatory and inhibitory neurons in the amygdala, which is involved in stress-induced emotional disorders. In addition, our data imply that glucocorticoid receptors, their intracellular signaling pathways and their targeted receptor-channels in the excitatory versus inhibitory neurons may be different, which is worthy to be deeply studied in the field of neuroscience.

Our studies also indicate that the different subcellular compartments of excitatory and inhibitory neurons express differential sensitivity to glucocorticoid action. For instance, the presynaptic terminals of glutamatergic neuron are sensitive to dexamethasone (Figs 4 and 5) but not their cell body (Fig 6). The presynaptic terminals of excitatory and inhibitory neurons are sensitive to both acute and chronic actions of dexamethasone, but the postsynaptic GABA_A receptors in the neurons are only sensitive to the chronic action of dexamethasone.

Fig 5. The acute application of dexamethasone decreases glutamate release from excitatory neurons of the amygdala. DEX was administrated by washing into the brain slices (25 μM). sEPSCs were recorded in the glutamatergic neurons of brain slices including the amygdala. A) shows sEPSCs in the control (left panel) and DEX-washing (right). The calibration bars are 10 pA versus 2 seconds (top traces) and 50 ms (bottoms). B) illustrates cumulative probability versus sEPSC amplitude in the control (blue symbols) and DEX-washing (red). C) illustrates cumulative probability versus inter-sEPSC intervals in the control (blue symbols) and DEX-washing (red).

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This incoordination among the subcellular compartments may lead to the complication of stress-induced emotional disorders [54].

By using mice with genetically YFP-labeled glutamatergic neurons and GFP-labeled GABAergic neurons in the amygdala, we are able to analyze cell-specific effect by glucocorticoids in their subcellular compartments and mutual interactions. The glucocorticoid-induced incompatibility among the subcellular compartments and the incoordination between GABAergic and glutamatergic neurons lead to imbalanced neural networks in the amygdala, which are the bases of stress-induced mood disorders.

**Methods and Materials**

All experiments were done in accordance with the guideline and regulation by the Administration Office of Laboratory Animals at Beijing China. All experimental protocols were approved by Institutional Animal Care Unit Committee in Administration Office of Laboratory Animals at Beijing China (B10831).

**Acute and chronic applications of dexamethasone**

In order to study the effects of dexamethasone (DEX), a potent agonist of glucocorticoid receptors, on the interactions between excitatory and inhibitory neurons, we applied C57 Thy1YFP/...
GAD1GFP mice whose GABAergic and glutamatergic neurons were genetically labeled by green fluorescent protein (GFP) and yellow fluorescent protein (YFP), respectively [55, 56]. In chronic application [52], DEX (40mg per kilogram of body weight) was intraperitoneally injected into these mice (postnatal day 20) once a day for one week. As body weights of these mice we used were about 20 grams on average, each mouse received 0.8 mg (i.e., 2 μM) DEX. This DEX concentration was close to its dosage that affected neuronal activities in a range of 0.5~5 μM [57]. In addition, we have measured the level of corticosterone in the mice that had received chronic unpredicted mild stress in day three about 300 ng/mL (i.e., 0.76 μM) [58]. Thus, DEX concentration used in our study was similar to that in chronic stress, and not over dosage. In acute application [21], DEX (25 μM) was washed onto the brain slices including the amygdala after the control data had been collected. This DEX concentration was based on a view that the concentration of corticosterone may be higher under acute stress than chronic stress [59]. In addition, we have found that 25 nM and 250 nM DEX are not effective on sEPSCs on GABAergic neurons in the brain slices. One could argue that dexamethasone is much stronger than corticosterone. In terms of pharmacological effects of dexamethasone versus corticosterone on biological processes, it is true that synthetic dexamethasone is stronger than natural corticosterone to affect the cells in the peripherals, such as anti-inflammation. However, the effects of dexamethasone and corticosterone on neuronal activities are variable and comparable in terms of their efficacies and concentrations [57, 60, 61].

Brain slices and neurons

To have more health brain cells for whole-cell recordings, we prepared cortical slices by the following procedures. The mice were anesthetized by isoflurane inhaling, and were infused by
the artificial cerebrospinal fluid (ACSF) and oxygenated (95% O\textsubscript{2} and 5% CO\textsubscript{2}) at 4°C into their left ventricles until the bodies became cold, in which the concentrations (mM) of the chemicals were 124 NaCl, 3 KCl, 1.2 Na\textsubscript{2}HPO\textsubscript{4}, 26 NaHCO\textsubscript{3}, 0.5 CaCl\textsubscript{2}, 4 MgSO\textsubscript{4}, 10 dextrose and 220 sucrose at pH 7.35. The mouse heads were immediately decapitated by guillotine and placed into this cold oxygenated ACSF for the brain isolation [31]. The cortical slices (300 μm) in coronal direction were cut by Vibratome in this cold oxygenated ACSF. They were held in another oxygenated ACSF (124 NaCl, 3 KCl, 1.2 Na\textsubscript{2}HPO\textsubscript{4}, 26 NaHCO\textsubscript{3}, 2 CaCl\textsubscript{2}, 2 MgSO\textsubscript{4}, 10 dextrose, and 5 HEPES, pH 7.35) at 25°C for 2 hours. Each slice was then placed into a submersion chamber (Warner RC-26G) that was perfused by the oxygenated ACSF at 31°C for the electrophysiological recordings [62–64]. The chemical reagents were from Sigma.

Whole-cell recording was done on GFP-labeled GABAergic and YFP-labeled glutamate neurons in the basal lateral area of the amygdala under DIC-fluorescent microscope (Nikon FN-E600, Japan). The wavelength at 488 nm excited the fluorescence of GFP-labeled neurons, and that at 575 nm excited the fluorescence of YFP-labeled neurons. The GABAergic neurons expressed fast spikes with less adaptation in their amplitudes and frequencies, the typical properties for the interneurons [65–69]. The glutamatergic neurons showed the pyramidal somata and spike adaptation [31].

Whole-cell recording and neuronal functions

The neurons were recorded by a MultiClamp-700B amplifier under voltage-clamp for their synaptic activity and the current-clamp for their intrinsic property. Electrical signals were inputted to the pClamp-10 (Axon Instrument Inc.) for data acquisition and analysis. An output bandwidth of this amplifier was set at 3 kHz [63]. The pipette solution to record excitatory activities included (mM) 150 K-gluconate, 5 NaCl, 5 HEPES, 0.4 EGTA, 4 Mg-ATP, 0.5 Tris-GTP, and 5 phosphocreatine (pH 7.35; [70, 71]). The solution to study inhibitory synapses contained (mM) 130 K-gluconate, 20 KCl, 5 NaCl, 5 HEPES, 0.5 EGTA, 4 Mg-ATP, 0.5 Tris-GTP and 5 phosphocreatine (pH 7.35; [46]). The pipette solutions were made freshly and filtered (0.1 μm). The osmolarity was 295–305 mOsmol and pipette resistance was 5–6 MΩ.

The functions of GABAergic neurons were studied including their active intrinsic properties and inhibitory outputs [72]. Their inhibitory outputs were assessed by recording spontaneous inhibitory postsynaptic currents (sIPSC) on glutamatergic neurons in the presence of 10 μM 6-Cyano-7-nitroquinoxaline-2,3-(1H,4H)-dione (CNQX) and 40 μM D-amino-5-phosphonovanolenic acid (D-AP5) in the ACSF to block ionotropic glutamatergic receptors. 10 μM bicuculline was washed onto the slices at the end of experiments for blocking sIPSCs to test that synaptic responses were mediated by GABA\textsubscript{A}R. The pipette solution with the high concentration of chloride ions makes reversal potential to be -42 mV. sIPSCs are inward when the membrane potential is held at -65 mV [46, 58, 73].

The functions of glutamatergic neurons were studied including the active intrinsic properties and excitatory outputs [72]. Their excitatory outputs were assessed by recording spontaneous excitatory postsynaptic currents (sEPSC) on GABAergic neurons in the presence of 10 μM bicuculline in the ACSF to block GABA\textsubscript{A}R [69, 72]. 10 μM CNQX and 40 μM D-AP5 were added into the ACSF at the end of experiments to test whether synaptic responses were mediated by glutamate receptor, which blocked sEPSCs in our studies.

The recording of spontaneous synaptic currents, instead of the evoked synaptic currents, is based on the following reasons. sEPSC and sIPSC amplitudes represent the responsiveness and the densities of postsynaptic receptors. The frequencies imply the probability of transmitter release from an axon terminal and the number of presynaptic axons innervated on the recorded neuron [74, 75]. These parameters can be used to analyze presynaptic and
postsynaptic mechanisms about the neuronal interaction. The evoked postsynaptic currents cannot separate these mechanisms. We did not add TTX in the ACSF to record miniature postsynaptic currents as we had to record neuronal excitability. As the frequency of synaptic activities was less than those of sequential spikes and the spontaneous spikes were never recorded on the neurons in our cortical slices, sIPSCs and sEPSCs were not generated from spontaneous action potential. The synaptic events in our recording are presumably miniature postsynaptic currents. This point is granted by a single peak of postsynaptic currents in our study [31].

Action potentials at the cortical neurons were induced by injecting the depolarization pulse. Their excitability was assessed by inter-spike intervals when the depolarization pulses were given [76]. We did not measure rheobase to show cellular excitability, as this strength-duration relationship was used to estimate the ability to fire single spike. We measured the capability of firing sequential spikes [62, 68].

Data were analyzed if the recorded neurons had the resting membrane potentials negatively more than -60 mV, and action potential amplitudes more than 90 mV. The criteria for the acceptance of each experiment also included less than 5% changes in resting membrane potential, spike magnitude, and input resistance throughout each recording. The series and input resistances in all neurons were monitored by injecting hyperpolarization pulses (5 mV/50 ms), and calculated by voltage pulses versus instantaneous and steady-state currents. The values in the amplitudes and inter-event intervals of sIPSCs and sEPSCs were read at 67% of cumulative probability (P_{0.67}) for their statistical comparisons [77]. It is noteworthy that the frequencies of sEPSCs and sIPSCs were applied to merit presynaptic transmitter release and the amplitudes of sEPSCs and sIPSCs were used to merit postsynaptic receptor functions [31].

Statistical analyses

The data of electrophysiological studies are presented as mean±SEM. Based on the principle of statistics, the paired-t-test is used for the comparisons before and after administering drugs, physical/chemical stimulations or molecular manipulations in a given group, such that paired t-test is used for the statistical comparison in neural activities before and after washing dexamethasone. One-way ANOVA is routinely used for the comparisons between groups, such as drugs versus controls, such that one-way ANOVA is used to make statistical comparison in neural activities between dexamethasone injection and control groups. The criterion for statistical significance is set at p<0.05.

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