Reduction in infarct size by local estrogen does not prevent autonomic dysfunction after stroke

TAREK M. SALEH,1,2 ALASTAIR E. CRIBB,1,2,3 AND BARRY J. CONNELL1
1Department of Anatomy and Physiology, 2Laboratory of Comparative Pharmacogenetics, Atlantic Veterinary College, and 3Prince Edward Island Health Research Institute, University of Prince Edward Island, Charlottetown, Prince Edward Island, Canada C1A 4P3

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Saleh, Tarek M., Alastair E. Cribb, and Barry J. Connell. Reduction in infarct size by local estrogen does not prevent autonomic dysfunction after stroke. Am J Physiol Regulatory Integrative Comp Physiol 281: R2088–R2095, 2001.—Systemic estrogen administration in male rats has been shown to normalize the autonomic dysfunction and reduce the infarct size after permanent middle cerebral artery occlusion (MCAO). Therefore, the present investigation determined if local microinjection of estrogen at the site of the infarct also promoted recovery of autonomic function and reduction of the infarct size. Experiments were done in anesthetized (thiobutabarbitol sodium; 100 mg/kg) male Sprague-Dawley rats instrumented to record baseline and reflex changes in cardiovascular and autonomic parameters. The right middle cerebral artery was permanently occluded using bipolar coagulation. Local microinjection of estrogen into the insular cortex before MCAO significantly reduced the infarct size but did not attenuate the MCAO-induced autonomic dysfunction. Injection of ICI-182,780 alone significantly increased infarct area; however, the greater infarct area was not associated with enhanced autonomic dysfunction. These results suggest that within the insula, endogenous estrogen activity can affect the extent of MCAO-induced cell death, but extracortical central nervous system sites may be responsible for mediating the beneficial effects of estrogen on the autonomic disturbances.

sympathetic nerve; parasympathetic nerve; baroreflex sensitivity; ICI-182,780; middle cerebral artery occlusion

THE MOST SIGNIFICANT CAUSE of mortality from cardiovascular disease in postmenopausal women is stroke (14). Almost 30% more postmenopausal women compared with age-matched men die as a result of stroke each year (5, 22). This mortality rate of postmenopausal women due to stroke is significantly reduced with the use of hormone replacement therapy (HRT; Ref. 22). Estrogen administration in postmenopausal women has been shown to lower blood pressure and heart rate (19), improve the reflex heart rate response to transient increases in blood pressure (11), reduce both the vascular α-adrenergic responsiveness (40) and the pressor response to mental stress (32), and enhance choline acetyltransferase activity at the heart (12).

These beneficial effects of estrogen are lost on termination of HRT (26). In premenopausal women, physiologically high concentrations of estrogen during the follicular phase of the menstrual cycle have been correlated with the greatest antifibrillatory protection (24). Subsequently, when estrogen levels are at their lowest in the luteal phase or similar to that of postmenopausal women, the induction of sympathetically mediated arrhythmias is significantly facilitated (24). Taken together with the fact that sympathoexcitation rarely occurs in premenopausal women or postmenopausal women on HRT (39), we hypothesize that estrogen has significant central actions that contribute to the maintenance of sympathovagal balance.

Both premenopausal women and postmenopausal women on HRT seem to be protected from stroke-induced cardiac arrhythmias and the resulting autonomic dysfunction (39). Such autonomic dysfunction is characterized by sympathoexcitation, leading to ventricular tachycardia and fibrillation within 1–2 h after the onset of clinical signs (acute phase after a stroke; Ref. 2). Cechetto and colleagues (8) developed a rat model of stroke involving the permanent occlusion of the middle cerebral artery (MCAO). As in the clinical situation, sympathetic tone and serum norepinephrine levels were significantly elevated within 30 min of the occlusion and lasted for the 6-h duration of the experimental time course (8).

Our laboratory has previously shown that estrogen acts centrally to improve sympathovagal balance by decreasing sympathetic tone and increasing parasympathetic tone in both male and female rats (33–35). In addition to enhancing sympathovagal balance, estrogen administered directly into several cardiovascular regulatory nuclei in the central nervous system significantly enhanced reflex autonomic function as measured by an increase in the sensitivity of the baroreceptor reflex (BRS; Ref. 32). The BRS is depressed after the onset of several cardiovascular pathologies (6, 7, 9, 12, 15–17, 20), including stroke (30, 41). Prior administration of systemic estrogen significantly reduces (~50%) the infarct size in various animal models of...
stroke, including permanent MCAO (3–5, 13, 31, 41, 42), and our laboratory has recently demonstrated that intravenous estrogen injection 30 min before MCAO in male rats completely reversed the resulting autonomic dysfunction (36). Estrogen administered 30 min after MCAO was associated with a recovery of autonomic tone and reflex function (BRS) within 90 min of injection; however, infarct size remained unchanged (36). This result suggested a degree of independence between the actions of estrogen on the infarct size and prevention of autonomic deficits after MCAO. Therefore, we could not be certain that the estrogen-induced prevention of autonomic dysfunction after MCAO was due solely to a reduced infarct size or the actions of estrogen elsewhere in the central nervous system. The current study was designed to determine if direct activation of estrogen receptors in the insular cortex by local injection of estrogen would reduce the infarct size due to actions of estrogen elsewhere in the central nervous system. The two could be dissociated.

MATERIALS AND METHODS

All experiments were carried out in accordance with the guidelines of the Canadian Council on Animal Care and were approved by the University of Prince Edward Island Animal Care Committee.

General surgical procedures. Experiments were performed on a total of 27 Sprague-Dawley male rats (Charles River, Montreal, Quebec, Canada) weighing 250–280 g. For all animals, food and tap water were available ad libitum. Rats were anesthetized with thiobutabarbital sodium (Inactin; RBI, Natick, MA; 100 mg/kg ip), which provided a stable plane of anesthesia for the duration of the experiment (no animals required anesthetic supplementation). A polyethylene catheter (PE-50; Clay Adams, Parsippany, NJ) was inserted into the right femoral artery to monitor blood pressure and heart rate, and a second catheter (PE-10) was inserted into the right femoral vein for the intravenous administration of drugs. Arterial blood pressure was measured with a pressure transducer (Gould P23 ID; Cleveland, OH) connected to a Gould model 2200S polygraph. Heart rate was determined from the pulse pressure using a Gould tachograph (Biotech). These parameters were displayed and analyzed by PolyviewPro/32 data-acquisition and -analysis software (Grass, Warwick, RI). An endotracheal tube was inserted, and animals were artificially ventilated with room air (Harvard rodent ventilator; 66 strokes/min; 2.5-ml tidal volume) and paralyzed with decamethonium bromide (Sigma-Aldrich, St. Louis, MO; 0.5 mg/kg iv). Body temperature was monitored with a digital rectal thermometer and maintained at 36 ± 1°C.

Autonomic nerve isolation and recording. All animals were instrumented to record changes in efferent parasympathetic and sympathetic nerve activities. To record efferent parasympathetic nerve activity, the left cervical vagus nerve was isolated through a midline cervical incision, placed on bipolar platinum recording electrodes, crushed distally, and secured in place with dental impression material (Basilex; Ash Temple, Bedford, NS). To record efferent sympathetic nerve activity, the right kidney was exposed through a retroperitoneal incision. With the aid of an operating stereomicroscope, a renal nerve branch was isolated from the surrounding tissue, and a bipolar platinum recording electrode was secured in place. The multifunit vagus and renal nerve activities were amplified by a Grass model P55 preamplifier with a 100-Hz to 3-kHz band pass and 60-Hz notch filter, displayed and analyzed using the PolyviewPro 32 data-acquisition and -analysis software. Animals were allowed to stabilize for 30 min after nerve isolation before drug injection or nerve activity measurements.

MCAOs. All animals were placed in a Kopf (Tujunga, CA) stereotaxic frame, and the right middle cerebral artery (MCA) was approached through a rostrocaudal incision in the skin and frontalis muscle at the level of bregma. The frontalis and temporalis muscles were then reflected anteriorly and posteriorly to expose the squamosal bone to the point where the zygoma fuses to the squamosal bone. A hand-held drill was used to make a burr hole in the rostrocaudal part of the squamosal bone, and the squamosal bone was removed to expose the MCA. The bent tip of a 25-gauge hypodermic needle was used to cut and retract the meninges over the MCA. The MCA was permanently occluded using bipolar electrical coagulation (Cameron-Miller, Chicago, IL) at three points. The first occlusion was made just dorsal to the rhinal fissure and the second occlusion was made just ventral to the bifurcation of the MCA to the frontal and parietal cortices, and the third occlusion was made just before the bifurcation of the MCA to the parietal cortex. In a sham-occlusion group, all surgical procedures described above were performed, except the MCA was not occluded.

Baroreflex testing, autonomic tone measurement, and drug injections. To determine the effect of MCAO on the reflexive changes in heart rate to baroreceptor activation, the baroreceptor reflex was evoked using bolus intravenous injections of the α-adrenergic receptor agonist phenylephrine hydrochloride (Sigma; n = 23). The peak amplitude of the resulting pressor and reflex bradycardia responses evoked by increasing doses of phenylephrine (0.025, 0.05, and 0.1 mg/kg) were plotted against each other. Regression lines were obtained by the least-squares method, and the slopes were used to provide an index of BRS. The slopes of the BRS curves and measures of both parasympathetic and sympathetic tone were determined 10 min before central drug administration and immediately before and 10, 30, 60, 90, 120, 180, and 240 min after either MCAO (n = 23) or sham (n = 4) treatment. Unilateral injections of 17β-estradiol (water-soluble form; 0.5 μM in 250 nl; Sigma-Aldrich) into the right (ipsilateral) insular cortex were carried out 10 min (n = 4) or 30 min (n = 4) before MCAO. To determine the specificity of the estrogen-induced effects on BRS and autonomic tone, the selective estrogen receptor antagonist ICI-182,780 (Tocris, Bolivia, MO; 0.9% saline and 0.03% ethanol; 1 μM in 250 nl; n = 4) or a cocktail of estrogen and ICI-182,780 (0.5 μM estrogen and 1 μM ICI-182,780 in 250 nl; n = 4) were made 10 min before MCAO in separate groups of rats (n = 4/group). In two additional groups (n = 4/group), physiological saline (0.9% in 250 nl) was injected into the insular cortex 10 min before either MCAO or sham (exposure and isolation of MCA) operation. In the final group (n = 3), estrogen (0.5 μM in 250 nl) was injected into the caudate putamen as a control for the spread of injectate. The doses of estrogen and ICI-182,780 were chosen on the basis of previous studies in our laboratory demonstrating that these doses produced either an optimal change in autonomic and cardiovascular parameters or maximally inhibited central estrogenic activity obtained after construction of a dose-response relationship (32, 37).

Histological procedures. Four hours after sham treatment or MCAO, the animals were transcardially perfused with PBS (0.1 M; 200 ml), and the brains were removed and sliced into 1-mm coronal sections using a rat brain matrix (Harvard Apparatus). Sections were then incubated in a 2% solution of...
2,3,5-triphenyltetrazolium chloride (TTC; Sigma-Aldrich) for 10 min and stored in 10% formalin. Infarct size was determined using a protocol similar to that used by Mathews and colleagues (21). Briefly, digital photographs of each section were taken to quantify infarct area using a computer-assisted imaging system (Bioquant; R & M Biometrics, Nashville, TN). Region of interest (absence of TTC staining within the prefrontal cortex) was outlined throughout the rostrocaudal extent, and the area was calculated after calibration of the image-analysis program. Verification of the insular cortex injections was made by examining the TTC-stained tissue under a dissecting microscope.

Data analysis. All data are presented as means ± SE and were analyzed by a two-way ANOVA for repeated measures followed by a Student-Newman-Keul post hoc analysis (SigmaStat and SigmaPlot; Jandel Scientific, Tujunga, CA). Statistical comparison of multiple regression line slopes was carried out with analysis of covariance. Differences in baseline or peak changes in renal or vagal nerve activity were determined using both an ANOVA and a Student t-test. In all cases, differences were considered significant if \( P \leq 0.05 \).

RESULTS

For all animals (\( n = 27 \)), rectal temperature remained constant at 36.5 ± 0.5°C compared with the prestroke or sham occlusion value throughout the experiment (\( P > 0.05 \)). Arterial blood gases were measured in three animals from samples taken before MCAO and at the end of the experiment (before death). No significant changes from pre-MCAO values for pH (7.35 ± 0.1), arterial \( P_{CO_2} \) (41 ± 0.4 mmHg), arterial \( P_{O_2} \) (99 ± 6 mmHg), or oxygen saturation (98.7 ± 0.5%) were measured (\( P > 0.05 \)). No significant baseline or reflex cardiovascular or autonomic changes were observed at any time during the experiment in sham-operated rats receiving central injections of saline (\( n = 4 \); Table 1; \( P > 0.05 \)) into the insular cortex. Because the effects of estrogen injection into the insular cortex 30 min before MCAO were not significantly different (\( P > 0.05 \)) from estrogen injected 10 min before MCAO (with the exception of infarct volume; see below), the cardiovascular and autonomic changes from these two groups were pooled; the combined results are described as the pre-MCAO group (MCAO + E; \( n = 8 \)).

Effect of stroke and drug injection into the insular cortex on baseline mean arterial pressure and heart rate. Before MCAO, the mean baseline arterial pressures and heart rates of all groups (\( n = 4 \)/group) were not significantly different from each other (\( n = 23; P > 0.05 \)). MCAO and central drug injection had no significant effect on either baseline mean arterial pressure or heart rate at any time point compared with measurements made before the MCAO (Figs. 1 and 2) or compared with sham-operated animals (\( n = 4 \)) at equivalent time points throughout the experiment (Figs. 1 and 2; \( P > 0.05 \)).

Effect of stroke and drug treatments on baseline autonomic tone. At all time points measured after MCAO regardless of drug treatment, renal sympathetic nerve activity (RSNA) was significantly elevated compared with sham-operated animals, from an average baseline value of 4.3 ± 0.2 to an average peak value of 6.6 ± 0.2 \( \mu V/s \) at 90 min after MCAO (Fig. 3A; \( P < 0.05 \)). Just before the termination of the experiment (4 h post-MCAO), average RSNA remained significantly elevated (5.1 ± 0.2 \( \mu V/s \); Fig. 3A; \( P < 0.05 \)) compared with either pre-MCAO values or the sham-operated animal average RSNA value at 240 min.

Compared with sham-operated animals for all drug injection groups, MCAO resulted in a significant decrease in vagal parasympathetic nerve activity (VPNA), from an average baseline value of 3.7 ± 0.2 to 1.8 ± 0.3 \( \mu V/s \) at 90 min after MCAO (\( P < 0.05 \)), and remained significantly attenuated at the termination of the experiment (2.8 ± 0.2 \( \mu V/s \) at 240 min post-MCAO; Fig. 3B; \( P < 0.05 \)).

Effect of stroke and drug treatments on BRS and autonomic function. Regardless of drug treatment, within 30 min after MCAO, average BRS was significantly depressed compared with sham-operated animals [from 0.48 ± 0.05 (\( n = 4 \)) to 0.27 ± 0.1 beats·min\(^{-1}·mmHg\)\(^{-1}\) at 90 min post-MCAO (\( n = 23 \); Fig. 4; \( P < 0.05 \)]. The decline in BRS value for all drug treatment groups continued and remained significantly depressed compared with both mean prestroke values or sham-operated values at the corresponding experimental time point (0.29 ± 0.05 at 240 min post-MCAO; Fig. 4; \( n = 23 \); \( P < 0.05 \)). The most profound effect was observed after the injection of ICI-182,780 into the insular cortex before MCAO (\( n = 4 \)), which resulted in a severe depression of BRS (0.07 ± 0.02 at 240 min post-MCAO) compared with MCAO group receiving either saline or estrogen (Fig. 4; \( P < 0.05 \)).

Table 1. Baseline cardiovascular and autonomic measurements obtained from sham-operated animals that received an intracortical injection of saline

<table>
<thead>
<tr>
<th>Time, min</th>
<th>10</th>
<th>30</th>
<th>60</th>
<th>90</th>
<th>120</th>
<th>180</th>
<th>240</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP, mmHg</td>
<td>94 ±11</td>
<td>97 ±10</td>
<td>94 ±11</td>
<td>100 ±11</td>
<td>100 ±7</td>
<td>96 ±7</td>
<td>94 ±6</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>335 ±14</td>
<td>361 ±24</td>
<td>336 ±12</td>
<td>345 ±21</td>
<td>347 ±14</td>
<td>368 ±41</td>
<td>346 ±12</td>
</tr>
<tr>
<td>VPNA, ( \mu V/s )</td>
<td>4.1 ±0.2</td>
<td>4.1 ±0.2</td>
<td>4 ±0.2</td>
<td>4 ±0.2</td>
<td>3.8 ±0.3</td>
<td>4 ±0.3</td>
<td>4 ±0.4</td>
</tr>
<tr>
<td>RSNA, ( \mu V/s )</td>
<td>3.7 ±0.2</td>
<td>4 ±0.2</td>
<td>4 ±0.2</td>
<td>4 ±0.15</td>
<td>4 ±0.3</td>
<td>4 ±0.3</td>
<td>4 ±0.4</td>
</tr>
<tr>
<td>BRS (slope)</td>
<td>0.5 ±0.04</td>
<td>0.5 ±0.03</td>
<td>0.5 ±0.01</td>
<td>0.5 ±0.04</td>
<td>0.5 ±0.02</td>
<td>0.4 ±0.03</td>
<td>0.5 ±0.05</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE; \( n = 4 \). All time points are relative to the time at which the middle cerebral artery was exposed and isolated. MAP, mean arterial pressure; HR, heart rate; VPNA, vagal parasympathetic nerve activity; RSNA, renal sympathetic nerve activity; BRS, baroreflex sensitivity.

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In addition to baroreflex sensitivity, reflex changes in autonomic tone (autonomic function) were tested in response to a rise in arterial pressure after phenylephrine injection (data not shown). Compared with pre-MCAO values, at all time points MCAO resulted in a significant attenuation in both the reflex increase in VPNA (from a 48 ± 5% change to a 35 ± 5% change; n = 4/group; P < 0.05) and reflex decrease in RSNA (from 55 ± 6% change to 25 ± 5% change; P < 0.05). These changes in reflex autonomic function returned to approximate pre-MCAO values at 180 min after MCAO (P > 0.05).

**Effect of stroke and drug treatments on infarct area.**
Infarct area after MCAO alone ranged from 26 to 39% of the total area of the right prefrontal cortex (n = 4; Fig. 5A). Estrogen injected 10 min before MCAO resulted in a significant reduction of this infarct area by 28 ± 12% (n = 4; Fig. 5A and B; P < 0.05). Estrogen injected 30 min pre-MCAO resulted in an even greater reduction of the infarct area (58 ± 12%; n = 4; Fig. 5B; P < 0.05). Coinjection of estrogen and ICI-182,780 10 min before MCAO did not significantly alter infarct size (110 ± 7%; n = 4; Fig. 5B; P > 0.05). Injection of ICI-182,780 alone before MCAO significantly increased the infarct area by 31 ± 6% (n = 4; Fig. 5A and B; P < 0.05).

**Histology.** Figure 5C shows the tip of the microinjection cannulas in the region of the insular cortex for both estrogen (10 min pre-MCAO) and ICI-182,780 groups. Also shown are the injection sites of estrogen made into the caudate putamen that did not result in any significant changes in the MCAO-induced autonomic dysfunction or infarct size (n = 3; P > 0.05). Injection sites for the two saline groups (MCAO + saline and sham operation + saline), coinjections of ICI-182,780 with estrogen 10 min before MCAO, or estrogen injected at 30 min before MCAO (n = 4/group) have been omitted from the diagram for clarity. However, all groups not shown in Fig. 5 had injection sites within the insular cortex.

**DISCUSSION**
In animal models of stroke, the promise of drug therapy is commonly judged from infarct size measure-
ments, assuming that a reduction in infarct size results in reduction of functional deficits. This study demonstrated that estrogen pretreatment significantly reduced the MCAO-induced infarct size but that this increased cell survival was not associated with a recovery of autonomic function. In fact, a 60% reduction in the size of the infarct area did not translate into preservation of function.

Previously, we demonstrated that the intravenous injection of estrogen before MCAO completely blocked the MCAO-induced autonomic dysfunction in addition to reducing the size of the infarct (36). However, when estrogen was injected 30 min after MCAO, it did not prevent the autonomic dysfunction from occurring but did result in a recovery of autonomic function within 90 min (36). In that group of animals, the size of the infarct area was not significantly different from MCAO alone. That result allowed us to hypothesize that estrogen was acting at extracortical sites to modulate the extent of autonomic dysfunction after MCAO. The results presented here further support this hypothesis because estrogen injected directly into the insular cortex reduced the MCAO-induced infarct size but did not result in a recovery of autonomic function. Current investigations in our laboratory are involved in antagonizing estrogen receptors in various autonomic regulatory nuclei to determine where in the central nervous system estrogen acts to protect against MCAO-induced autonomic dysfunction.

A dissociation between infarct size and the degree of functional deficit was also observed in a study in which isradipine was used as the cytoprotectant in a model of permanent MCAO. The authors reported a significant reduction in the infarct size (40%) with no significant improvement in the functionality of neurons during the acute poststroke phase (29). The apparently intact somatosensory cortex in isradipine-treated animals responded to orthodromic electrical stimulation similarly to vehicle-treated controls with an infarcted cortex (29). Similar neuroprotection was observed in a study in which fetal neocortical grafts placed in the ischemic zone after MCAO resulted in a significant decrease in the infarct size, but again did not affect functional recovery as assessed by behavioral testing (18).

In some studies, lesion size has been correlated with functional deficit. During model development and behavioral assessment of focal, transient cerebral ischemia in rats, a positive correlation between infarct volume and clinical behavioral score was observed (28). Infarct volume also correlated with the number of vessels occluded and the duration of MCAO occlusion (28). This study demonstrated that cell death was indeed correlated to the duration of oxygen and glucose deprivation, resulting in greater behavioral deficits. In a similar study, systemic administration of nicotinamide (23) reduced infarct volume by 46% and resulted in improved neurological outcomes (sensory and motor behavior). Our previous results showed that systemic administration of estrogen reduced the infarct size and prevented autonomic dysfunction (36). Therefore, these studies suggested that increased cell survival would be correlated with greater functional recovery.

A study done by Cheung et al. (10) demonstrated that significant neurochemical changes are observed in extracortical autonomic and cardiovascular regulatory nuclei after permanent MCAO. In fact, these authors found very subtle changes in the neurochemistry of the

Fig. 4. Slope of the regression line (baroreflex sensitivity; BRS) in sham-operated animals and MCAO groups of animals receiving either saline, estrogen, or ICI-182,780. Dashed line, time at which the MCA was occluded or exposed in sham-operated animals. All data points after MCAO are significantly different from sham-operated animal data at similar times (P < 0.05; analysis of covariance).
insular cortex, particularly in the region of the ischemic zone and penumbra. This suggests that systemic administration of neuroprotective agents, such as nicotinamide and estrogen, employed in a stroke model could be acting at extracortical central nervous system sites, leading to improved functional outcomes.

The fact that both estrogen-receptor subtypes (ER-α and ER-β) are found in particularly high concentrations within the agranular and dysgranular insular cortex (38) may help provide insight into the results from the estrogen receptor antagonist group in the current study. In this group (ICI-182,780 alone), infarct size was significantly greater than in the MCAO and saline group, but autonomic and cardiovascular dysfunction were similar. We expected to see a greater autonomic dysfunction with the increased cell death in the cortex; however, this was not the case. Although in the opposite direction as neuroprotective agents, again, a correlation between changes in infarct size and function was not observed. The fact that ICI-182,780 injections into the insular cortex resulted in a significant increase in infarct size does suggest a role for endogenous estrogen levels within the insula in protecting against ischemic cell death in male rats.

Perspectives

In most previous studies concerning the effects of ischemia on cognition, there has not been an attempt to correlate pathology with functional outcome measurements. In many studies, the degree of damage to the brain measured by volume of infarct after an ischemic insult is not provided (1, 25). The results presented here, taken together with our previous findings, dem-
onstrate the need for functional testing (autonomic and cardiovascular, behavioral, or sensory) to provide evidence on the relationship between cell survival and the recovery of function in models of neuroprotection after MCAO. On the basis of the evidence available to date, there is probably not a critical level of cell survival that is required before recovery of function (autonomic, motor, or sensory) can be measured. However, the reverse may hold true in that a minimal amount of cell death is all that is needed to result in significant functional deficits. It may require a minimum amount of cell death in the insular cortex to produce an abnormal output from this region to alter the function of subcortical autonomic nuclei.

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