ADRENAL STEROIDS AND N-METHYL-D-ASPARTATE RECEPTOR ACTIVATION REGULATE NEUROGENESIS IN THE DENTATE GYRUS OF ADULT RATS THROUGH A COMMON PATHWAY

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Abstract--Adrenal steroids and N-methyl-D-aspartate receptor activation have both been shown to regulate the rate of proliferation of granule neuron progenitor cells in the dentate gyrus of adult rats [Cameron H. A. and Gould E. (1994) Neuroscience 61, 203–209; Cameron H. A. et al. (1995) J. Neurosci. 15, 4687–4692]. Parallels between the actions of these two factors suggest that they may regulate cell division through a common pathway. This hypothesis was tested by altering both of the factors simultaneously and determining whether the effects were additive.

The results of this study demonstrate that alterations in N-methyl-D-aspartate receptor activation block the effects of corticosterone level on cell proliferation; N-methyl-D-aspartate blocks the adrenalectomy-induced increase in [H]thymidine-labelled cell density in the dentate gyrus, whereas the N-methyl-D-aspartate receptor antagonist dizocilpine maleate (MK-801) prevents the corticosterone-induced decrease in proliferating cells. This finding suggests that adrenal steroids and N-methyl-D-aspartate receptor activation regulate granule cell production in the adult rat dentate gyrus through a common pathway and that N-methyl-D-aspartate receptor activation operates downstream of corticosterone in this pathway.

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Key words: cell division, granule cell, dentate gyrus, adrenal steroids, NMDA receptors.

An increasing body of evidence demonstrates that granule neurons of the dentate gyrus, unlike neurons in most mammalian brain regions, continue to be produced into adulthood. Within a few weeks of division, cells generated in the dentate gyrus of adult rats can be identified as neurons on the basis of their location in the granule cell layer, their morphological characteristics, and their expression of the neuronal marker, neuron-specific enolase. Additionally, these cells have been found to have synapses on their cell bodies and dendrites and extend axons into the mossy fibre pathway.

Neurogenesis in the dentate gyrus of adults is likely to be a common characteristic of most mammalian species, since it has been reported to occur in mice, meadow voles, rats, tree shrews and marmoset monkeys.

Several previous studies have demonstrated that both adrenal steroids and N-methyl-D-aspartate (NMDA) receptor activation regulate the production of new neurons in the dentate gyrus of the rat during development and in adulthood. In the adult, adrenalectomy and blockade of NMDA receptors both result in increases in the production of granule neurons while increased corticosterone and NMDA receptor activation diminish proliferation of granule cell precursors.

Collectively, these observations suggest intriguing parallels between the actions of these two factors and are consistent with the possibility that adrenal steroids and excitatory input and/or NMDA receptor activation regulate the production of neurons by acting through a common pathway. Low levels of adrenal steroids following adrenalectomy in adulthood may stimulate cell proliferation by decreasing excitation, whereas high levels of adrenal steroids may result in increased excitation, which would decrease the rate of cell proliferation. The observations that increased glucocorticoids have been shown to stimulate glutamate release and alter the expression of NMDA receptors in the hippocampus are consistent with this hypothesis. To determine whether corticosterone and NMDA receptor activation regulate cell division in the dentate gyrus through a common pathway, we altered both of these factors simultaneously to determine if their effects on the number of proliferating cells were additive, implying independent mechanisms, or if one factor blocked the effect of the other, indicating a common pathway.
Experiments were known from previous experiments to involve the interaction between adrenal steroids and NMDA receptor activation in the regulation of cell division in the dentate gyrus, the effect of acute changes in adrenal steroid levels and NMDA receptor activation combined was compared to the effect of altering either adrenal steroid levels or NMDA receptor activation alone. Manipulations that drive cell proliferation in opposite directions were combined in order to avoid a floor or ceiling effect, i.e. reaching a minimum or maximum level of cell division that could not be further decreased or increased by the second factor. It was also important for the effects of the factors to be similar in magnitude, in order to exclude the possibility that a large effect of one factor might prevent the effect of the other factor from being observed; the doses of corticosterone, NMDA, and dizocilpine maleate (M K-801) chosen for these experiments were known from previous experiments to have effects on \[3H\]thymidine-labelled cell density that were similar in magnitude to each other and to the effect of adenectomy. In the first experiment, injection of the adrenal steroid, corticosterone, which suppresses cell proliferation, was combined with injection of the NMDA receptor antagonist, M K-801, which enhances cell proliferation. In the second experiment, removal of adrenal steroids by adenectomy, which enhances cell proliferation, was combined with injection of the NMDA receptor antagonist, NMDA, which suppresses cell proliferation.

Combined adrenal steroid removal and N-methyl-D-aspartate receptor activation. The number of proliferating cells in the dentate gyrus was compared in four treatment groups: i) adenectomy, ii) NMDA receptor activation, iii) adenectomy and NMDA receptor activation, and iv) control. Treatments were given according to the following time course (Fig. 1B). Rats in groups 1 and 3 were subjected to bilateral adrenalectomy, performed using aseptic procedures under Metofane anesthesia. The rats in the other two groups received sham operations at this time. The animals were allowed to recover from surgery for five days; during this period, adenolexedemated rats were given drinking water containing sodium chloride (0.9% w/v), to maintain salt balance, and corticosterone (25 µg/ml with ethanol 0.15% v/v), to allow time for recovery without producing a chronic period of low corticosterone. This replacement dose of corticosterone results in blood levels that are within the lower range of normal levels and prevents granule cell death in the dentate gyrus. After the five day recovery period, the drinking water with corticosterone was removed in the early morning (lights on) and replaced with physiological saline; corticosterone levels drop dramatically within 6 h after removal of adrenal steroids. Two hours later, the rats in groups 2 and 3 received a single i.p. injection of the NMDA receptor agonist NMDA (30 mg/kg, in saline). The rats in the other two groups received saline injections at this time. The rats in all four groups received a single intraperitoneal injection of 5.0 µCi \[3H\]thymidine/g body weight (New England Nu clear, specific activity 80 Ci/mmol), a marker of DNA synthesis that labels proliferating cells and their progeny. All rats were perfused 2 h after \[3H\]thymidine injection, a survival time that is adequate for the uptake of \[3H\]thymidine by cells synthesizing DNA but not sufficient for the completion of mitosis or migration, thus identifying the location of the progenitor population. The brains were processed for autoradiography combined with Nissl staining as described below.

**EXPERIMENTAL PROCEDURES**

Animal treatments

Adult (> two-months-old) male Sprague-Dawley rats (Charles River) were used for both experiments (n = 5 in each group). All rats were group-housed and provided with unlimited access to food and water. In order to investigate the interaction between adrenal steroids and NMDA receptor activation in the regulation of cell division in the dentate gyrus, the effect of acute changes in adrenal steroid levels and NMDA receptor activation combined was compared to the effect of altering either adrenal steroid levels or NMDA receptor activation alone. Manipulations that drive cell proliferation in opposite directions were combined in order to avoid a floor or ceiling effect, i.e. reaching a minimum or maximum level of cell division that could not be further decreased or increased by the second factor. It was also important for the effects of the factors to be similar in magnitude, in order to exclude the possibility that a large effect of one factor might prevent the effect of the other factor from being observed; the doses of corticosterone, NMDA, and dizocilpine maleate (M K-801) chosen for these experiments were known from previous experiments to have effects on \[3H\]thymidine-labelled cell density that were similar in magnitude to each other and to the effect of adenectomy. In the first experiment, injection of the adrenal steroid, corticosterone, which suppresses cell proliferation, was combined with injection of the NMDA receptor antagonist, M K-801, which enhances cell proliferation. In the second experiment, removal of adrenal steroids by adenectomy, which enhances cell proliferation, was combined with injection of the NMDA receptor antagonist, NMDA, which suppresses cell proliferation.

Combined N-methyl-D-aspartate receptor blockade and increased corticosterone level. The density of \[3H\]thymidine-labelled cells in the dentate gyrus was compared in four treatment groups: i) NMDA receptor blockade, ii) increased corticosterone level, iii) NMDA receptor blockade and increased corticosterone level, and iv) control. Treatments were given according to the following time course (Fig. 1A). Rats in groups 1 and 3 each received a single intraperitoneal (i.p.) injection of the specific noncompetitive NMDA receptor antagonist, M K-801 at a dose 10.0 mg/kg in saline) that has been shown to block NMDA receptors and increase cell proliferation in the dentate gyrus. The rats in the other two groups received an i.p. injection of saline at this time. One hour later, rats in groups 2 and 3 received a single subcutaneous injection of corticosterone in sesame oil at a dose 40 mg/kg previously shown to decrease cell proliferation in the dentate gyrus. The rats in the other two groups received no injections at this time, as subcutaneous oil injections have been found to decrease cell proliferation in the dentate gyrus, presumably by producing an endogenous rise in corticosterone due to the stress of injection. One hour after the time of corticosterone injection, rats in all four groups received a single i.p. injection of \[3H\]thymidine (5.0 µCi/g body weight, New England Nu clear, specific activity 80 Ci/mmol), a marker of DNA synthesis that labels proliferating cells and their progeny. All rats were perfused 2 h after \[3H\]thymidine injection, a survival time that is adequate for the uptake of \[3H\]thymidine by cells synthesizing DNA but not sufficient for the completion of mitosis or migration, thus identifying the location of the progenitor population. The brains were processed for autoradiography combined with Nissl staining as described below.

**Histological procedures**

All rats were deeply anaesthetized with M etofane and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer. The sections were mounted onto gelatinized slides, dried, dipped in photographic emulsion (NTB-2, Kodak), and stored in the dark at 4°C for two weeks. The slides were then developed in Dektol (Kodak), rinsed in water, fixed in Polymax T (Kodak), rinsed in water, Nissl stained with Cresyl Violet, dehydrated, cleared in Amiclear, and coverslipped with Permount.

**Data analysis**

The slides were coded prior to quantitative analysis and the code was not broken until the analysis was completed. Neuronatomically matched sections were selected from the middle portion of the dentate gyrus, where the suprapyramidal and infrapyramidal blades are joined at the crest and horizontally oriented beneath the corpus callosum, approximately Bregma -3.3 to -4.8. A defined rostrocaudal level was chosen in order to avoid any quantitative regional differences in cell proliferation that may exist. For each section, \[3H\]thymidine-labelled cells in the granule cell layer and in the hilus were counted using a light microscope (1000 ×). A cell was considered labelled if it had greater...
Adrenal steroids regulate neurogenesis via NMDA receptors

RESULTS

The effects of increased corticosterone level and N-methyl-D-aspartate receptor blockade on cell proliferation

In order to investigate the hypothesis that blockade of NMDA receptors prevents the corticosterone-induced inhibition of cell proliferation in the dentate gyrus, we compared the number of proliferating cells in the dentate gyrus of adult rats treated with both corticosterone and M K -801 to rats treated with either corticosterone or M K -801 alone and to rats not treated with either drug. In all groups, the \([^3H]\)thymidine-labelled cells/10^6 µm^2. Means of these densities were determined for each animal from at least four sections per brain. The data were subjected to one-way ANOVA followed by Tukey HSD post hoc comparisons.

![Image](image_url)

**Table 1. The effects of increasing corticosterone level and/or decreasing N-methyl-D-aspartate receptor activation on the density of dividing cells in the dentate gyrus.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Density of Dividing Cells (mean ± S.E.M., each obtained from five animals)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No injection</td>
<td>16.74 ± 0.47</td>
</tr>
<tr>
<td>Corticosterone injection</td>
<td>0.815 ± 0.237*</td>
</tr>
<tr>
<td>Corticosterone injection</td>
<td>8.236 ± 0.795*</td>
</tr>
</tbody>
</table>

†Indicates significant difference (P < 0.05) from control group (saline injection-no injection at the time of corticosterone injection) with Tukey HSD post hoc test.

The effects of adrenalectomy and N-methyl-D-aspartate receptor activation on cell proliferation

A second experiment with a converse design was performed to corroborate the findings of the first experiment. In this experiment, rats that had both decreased corticosterone levels and increased NMDA receptor activation levels were compared to rats with either decreased corticosterone levels, increased NMDA receptor activation levels, or controls. Short-term decreases in circulating corticosterone levels, produced by briefly removing corticosterone from the drinking water of adrenalectomized rats, resulted in a large (≈300% of control value) and statistically significant increase in the density of \([^3H]\)thymidine-labelled cells in the dentate gyrus compared to sham-operated rats (Table 2). A single injection of NMDA produced a large and statistically significant decrease (to ≈10% of control) in \([^3H]\)thymidine-labelled cell density compared to saline injection (Table 2). Combining corticosterone removal with NMDA injection produced nearly the same density of \([^3H]\)thymidine-labelled cells (≈15% of control) as NMDA injection alone (Table 2). As previously observed with acute M K -801 treatment or adrenalectomy with corticosterone replacement, the mean area of the dentate gyrus was not significantly different in any of the treatment groups, indicating that changes in \([^3H]\)thymidine-labelled cell density reflect changes in the total number of \([^3H]\)thymidine-labelled cells.

**Table 2. The effects of decreasing adrenal steroid levels and/or increasing N-methyl-D-aspartate receptor activation on the density of dividing cells in the dentate gyrus**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Density of Dividing Cells (mean ± S.E.M., each obtained from five animals)</th>
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<tbody>
<tr>
<td>Sham operation</td>
<td>4.729 ± 0.493</td>
</tr>
<tr>
<td>A drenalectomy</td>
<td>8.236 ± 1.717*</td>
</tr>
<tr>
<td>N M D A injection</td>
<td>0.204 ± 0.032*</td>
</tr>
</tbody>
</table>

†Values indicate mean number of \([^3H]\)thymidine-labelled cells/10^6 µm^2 ± S.E.M., each obtained from five animals.

*Indicates significant difference (P < 0.05) from control group (sham operation+saline injection) with Tukey HSD post hoc test.

**Table 3. The effects of using NMDA receptors to inhibit cell proliferation in the dentate gyrus**

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\[\text{Density of }[^3H]\text{thymidine-labelled cells/10}^6\text{µm}^2\]

**Table 4. The effects of using NMDA receptors to inhibit cell proliferation in the dentate gyrus**

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DISCUSSION

The results of this study demonstrate that alterations in NMDA receptor activation block the effects of corticosterone on cell proliferation; NMDA blocks the adrenalectomy-induced increase in cell proliferation in the dentate gyrus, whereas MK-801 prevents the corticosterone-induced decrease in proliferating cells.

The speed of the effects on cell division

Previously, we have observed increased cell proliferation in the dentate gyrus several days after adrenalectomy.6,16 By replacing corticosterone in the drinking water of adrenalectomized rats until 3 h before [3H]thymidine injection, the current study demonstrates that a drop in corticosterone levels stimulates cell proliferation within as little as 3–5 h. An effect of increased corticosterone was seen in this experiment within 1–3 h of administration ([3H]thymidine injection occurred 1 h after corticosterone injection, and animals were perfused 2 h after [3H]thymidine injection); this extends the earlier finding that 24 h of increased corticosterone levels results in a decrease in [3H]thymidine-labelled cells.6 The effects of NMDA receptor activation or blockade on [3H]thymidine-labelled cell density in the dentate gyrus also occur rapidly, within 1–3 h (current study). It is not known whether these increases and decreases in the numbers of [3H]thymidine-labelled cells in the dentate gyrus reflect changes in the size of the population of proliferating cells and/or changes in the rate of progression through the cell cycle. However, the rapid entry of these cells into S-phase following decreased corticosterone or NMDA receptor blockade suggests that the effects may be on cells that are already proliferating, since it is believed that moving cells from G0 to quiescent phase to S-phase takes longer than moving from G1 to S-phase and requires several hours, at least in vitro.38

The direct signal to proliferating cells

It is not currently known which cells corticosterone and NMDA agonists/antagonists act on to produce changes in cell division. It appears, however, that these factors do not act directly on the proliferating progenitors, since very few dividing cells are immuno-reactive for either of the adrenal steroid receptors, Type I (mineralocorticoid) receptors and Type II (glucocorticoid) receptors,6 or for N R 1,7 a subunit of the NMDA receptor believed to be found in all NMDA receptors with functional calcium channels.33 However, it is conceivable that glutamate could act directly on the proliferating precursors through an immature form of NMDA receptors that does not act through opening of a calcium channel, or through a different glutamate receptor, which has been described as having similar ligand binding characteristics to the NMDA receptor but without any NR1 subunits.34 Another possibility is that corticosterone and glutamate may act on cells close to the granule cell precursor, which in turn signal the precursor cells to enter S phase or stop at the G1/S border by altering the availability of a mitogenic factor, such as basic fibroblast growth factor, transforming growth factor-β, or transforming growth factor-α, or transforming growth factor-β, all of which have mitogenic effects in several systems12,13,38,41,47 and are expressed in the dentate gyrus.20,42,48,51 The finding that epidermal growth factor receptors can be immunohistochemically identified on a proportion of dividing cells in the dentate gyrus supports the idea that this mitogen could act as the direct signal for proliferation.

The common pathway for corticosterone and N-methyl-D-aspartate receptor activation

The finding that the effects of corticosterone on cell division can be blocked by treatments that activate or inactivate NMDA receptors suggests that corticosterone and NMDA receptor activation alter cell division in the adult rat dentate gyrus through a common pathway and, additionally, that NMDA receptor activation acts downstream of corticosterone in this common pathway.

A previous study has shown that lesioning the entorhinal cortex, which provides the major glutamatergic input to the dentate gyrus, increases cell division in the dentate gyrus in a manner that is quantitatively similar to the increase observed following NMDA receptor blockade with MK-801 or CGP 37849.6 This result suggests that the important NMDA receptors in the pathway regulating dentate gyrus cell division are the NMDA receptors on the dendrites of mature granule cells, which receive glutamatergic input from the entorhinal cortex stellate cells via the perforant path. A second study, showing that a small lesion in the granule cell layer of the dentate gyrus increases the proliferation of nearby granule cell precursors,38 also suggests that mature granule cells play a role in regulating the division of immature progenitors. Mature granule cells may integrate the signals from excitatory input and corticosterone and subsequently increase or decrease the release of a mitogen that directly regulates the proliferation of the granule cell precursors.

There are several ways in which corticosterone could act upstream of NMDA receptor activation. Corticosterone could bind to receptors on the stellate cells, which have both mineralocorticoid and glucocorticoid receptors (Gould E., unpublished observations), and act anatomically upstream in the circuit from the granule cell NMDA receptors by increasing the release of glutamate by the stellate cells onto the granule cells. The finding that glucocorticoids increase the release of glutamate in the hippocampus is consistent with this idea. A second
possibility is that corticosterone may bind directly to mature granule cells, which also have mineralocorticoid and glucocorticoid receptors, and act upstream from the NMDA receptors in the mitogen expression pathway, e.g., corticosterone could decrease transcription of a growth factor while NMDA receptor-mediated calcium entry could alter the secretory trafficking of the factor. This model, however, could only work for short periods of NMDA receptor activation, until the available pool of growth factor is completely released; production of more growth factor would then be required in order to see a continued effect of secretory trafficking. A third possibility is that corticosterone, or one of its metabolites, might directly modulate the NMDA receptor channel; glucocorticoids have been shown to enhance excitotoxic damage mediated by NMDA receptors, but not by non-NMDA receptors, suggesting that they may not act by simply increasing glutamate release, and steroid hormones related to corticosteroids, such as pregnenolone, have been shown to directly potentiate NMDA receptor channel opening and NMDA-induced elevations in intracellular Ca++. Corticosterone and NMDA receptor activation, which we have found to regulate granule cell precursor proliferation through a common pathway, also appear to be linked in some learning paradigms and in dentate gyrus granule cell long-term potentiation. These parallels suggest that continuing production of new granule neurons may play an important role in the normal functioning of the dentate gyrus.

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