Activation of the AT₂ Receptor of Angiotensin II Induces Neurite Outgrowth and Cell Migration in Microexplant Cultures of the Cerebellum*

(Received for publication, April 19, 1999, and in revised form, August 6, 1999)

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Microexplant cultures from three-day-old rats were used to investigate whether angiotensin II (Ang II), through its AT₁ and AT₂ receptors, could be involved in the morphological differentiation of cerebellar cells. Specific activation of the AT₂ receptor during 4-day treatment induced two major morphological changes. The first was characterized by increased elongation of neurites. The second change was cell migration from the edge of the microexplant toward the periphery. Western blot analyses and indirect immunofluorescence studies revealed an increase in the expression of neuron-specific βIII-tubulin, as well as an increase in expression of the microtubule-associated proteins tau and MAP2. These effects were demonstrated by co-incubation of Ang II with 1 μM DUP 753 (AT₁ receptor antagonist) or with 10 nM CGP 42112 (AT₂ receptor agonist) but abolished when Ang II was co-incubated with 1 μM PD 123319 (AT₂ receptor antagonist), indicating that differentiation occurs through AT₂ receptor activation and that the AT₁ receptor inhibits the AT₂ effect. Taken together, these results demonstrate that Ang II is involved in cerebellum development for both neurite outgrowth and cell migration, two important processes in the organization of the various layers of the cerebellum.

A large number of studies indicate that the hormone angiotensin II (Ang II) and its receptors are present in the brain (1, 2). As in the periphery, the AT₁ receptor exhibits a high affinity for the nonpeptidic antagonist DUP 753 (Losartan), whereas the AT₂ receptor has a high affinity for the antagonist PD 123319 and the agonist CGP 42112 (1, 2). Although the AT₁ receptors are detected in areas involved in the regulation of blood pressure, hydromineral balance, and thirst, no central receptors are detected in areas involved in the regulation of blood pressure, hydromineral balance, and thirst, no central function has yet been attributed to the AT₂ receptor. This receptor is highly expressed in the inferior olive, locus coeruleus, thalamic nuclei, medial geniculate nuclei, and the molecular layer of the cerebellum (3–5).

Although several studies have been conducted on the short term effect of AT₂ receptor activation on intracellular events, a few studies focused on the physiological function of the AT₂ receptor. One well described function is its antagonistic action on cellular growth induced by neurotrophic factors (nerve growth factor) (6, 7) or by the AT₁ receptor of Ang II (8–10). Another function recently described for the AT₂ receptor is a role in programmed cell death (11, 12). Interestingly, although the expression of the AT₁ receptor either remains stable or increases with development in rats, the expression and density of the AT₂ receptor decrease dramatically with maturation from fetal to neonatal to adult, both at the periphery and in several brain nuclei (13–16). This high and transient expression of the AT₂ receptor in fetal tissues suggests that it may play a specific role during development and cellular differentiation (7, 11, 17, 18). Indeed, in a previous study, using neuroblastoma × glioma hybrid NG108–15 cells, we have shown that a 3-day treatment with Ang II or CGP 42112 induced neurite outgrowth characterized by an increase in the level of polymerized tubulin and in the association of the microtubule-associated protein MAP2c with microtubules (18). A similar effect was observed in PC12W cells, with a decrease in another microtubule-associated protein, MAP1B (7), and an increase in the association of MAP2 with microtubules (19) as well as with an increase in the neurofilament middle subunit NF-M (20).

Neuronal development and differentiation of the cerebellum involve several steps including proliferation (in the ventricular zone), migration (through the ventricular zone to the cortical zone), and finally either neurite extension or apoptosis, once the cells have reached their specific location (21, 22). Each of these steps is controlled by several local environmental cues, such as components of the extracellular matrix and cell adhesion molecules (23, 24). However, it is clear that the molecular identity of all of the regulators is yet to be determined. In particular, despite the studies conducted on neuronal cell lines (see above), so far there have been no studies on the long term effect of Ang II on either neuronal differentiation or neuronal development of specific brain areas. Therefore, the aim of the present study was to investigate the role of the AT₁ and AT₂ receptors on neurite outgrowth and on the pattern of expression of tubulin as well as of tau and MAP2, two important microtubule-associated proteins, in primary cultures of neonatal rat cerebellar neurons. The cerebellum was chosen for several reasons: 1) Ang II as well as both AT₁ and AT₂ receptors are present throughout cerebellar development (3, 14, 25, 26), suggesting a role for Ang II in this brain structure (26, 27). 2) Differentiation of cerebellar granule cells occurs mostly during the postnatal period (21, 28), implicating both cell migration from the molecular layer to the Purkinje cell layer and neurite elongation (22). 3) The juvenile and adult forms of tau and
MAP2 are differentially expressed during maturation of this brain area and may serve as markers of the state of neuronal maturation (29, 30).

**EXPERIMENTAL PROCEDURES**

**Chemicals**—The chemicals used in the present study were obtained from the following sources. Angiotensin II was from Bachem (Marina Delphen, CA); glutamine, neurobasal medium, and B27 supplement were from Life Technologies, Inc. CGP 42112, DUP 753, and PD 123319 were synthesized at Ciba-Geigy, Basel, Switzerland, and anti-mouse IgG-fluorescein was from Amersham Pharmacia Biotech. Monoclonal anti-β-tubulin and enhanced chemiluminescence detection system were from Roche Molecular Biochemicals; anti-GFAP, anti-neurofilament NE-14, anti-IIH-tubulin, and the monoclonal antibody HM-2, which recognizes all MAP2 isoforms, were purchased from Sigma. Monoclonal tau antibody SE2 was kindly provided by Dr. Kenneth Kosik (Center for Neuroscience Diseases, Brigham and Women’s Hospital and Harvard Medical School, Boston, MA), and tau antibody was kindly provided by Dr. Lester Binder (Department of Cell Biology, University of Alabama at Birmingham, AL). Ang II was iodinated in the laboratory of Dr. Gaeten Guillemette (Department of Pharmacology, University of Sherbrooke, PQ, Canada). Vectashield mounting medium was from Vector Laboratories (Burlingame, CA). All other chemicals were of A grade purity.

**Preparation of Cell Cultures**—Primary cultures of mixed cerebellar cells were prepared from the methodology described by Moonen et al. (31), with the following modifications. Cerebellei (10–12-culture) from Long Evans rats at postnatal day 3 were isolated and mechanically dissociated in neurobasal medium supplemented with B27 and 0.5 mM glutamine. The suspension was centrifuged at 100 × g for 10 min at room temperature. The cell pellet was suspended in the same medium and plated at a density of 1.5 × 10^5 cells/35-mm Petri dish, precoated with poly-L-lysine. Cells were grown in a humidified atmosphere of 95% air, 5% CO₂, at 37 °C. 24 h after plating, cells were treated for 4 consecutive days without (control cells) or with CGP 42112, the AT₂ receptor agonist, or with Ang II (100 nM) alone or in the presence of AT₂ receptor antagonist (1 μM), or PD 123319, an AT₁ receptor antagonist (1 μM) and were used on the 6th day.

Ang II binding studies were conducted on cells cultured for 6 days according to the methodology previously described (18). Density of Ang II receptor subtypes were identified as total binding sensitivity toward the AT₁ and AT₂ receptor analogs.

**Preparation of Microtubule Proteins**—Preparations enriched in microtubules were obtained from cells cultured for 6 days in 35-mm Petri dishes as described by Solomon (32) with slight modifications described previously (18). The cells were pretreated with 1 μM Taxol (Sigma) for 2 h before extraction of microtubules. At this concentration Taxol stabilizes microtubules without promoting polymerization. The culture medium was then aspirated and replaced by PM2G buffer (0.1 M PIPES, 2 mM glucose, 5 mM MgCl₂, 2 mM EGTA, 0.04 trypan blue unit/ml, 0.02% Triton X-100, 1 mM benzamidine, pH 6.9) containing Taxol (1 μM). After collection and centrifugation (1,000 × g for 5 min at 37 °C), the cell pellet was extracted with PM2G buffer containing 1% Nonidet P-40 and 1 μM Taxol (15 min incubation at 37 °C). After centrifugation, the resulting pellet (containing microtubules) was solubilized in electrophoresis sample buffer (Tris buffer 62.5 mM, pH 6.8 containing 2% SDS (w/v), 10% glycerol (w/v), 0.1% β-mercaptoethanol) and heated to 100 °C for 5 min. After centrifugation, the supernatant was added to 20 °C. For total cell extracts, cells grown in 35-mm Petri dishes were washed twice with HBS buffer 13. 3m NaCl, 3.5 mM KCl, 1.8 mM CaCl₂, 0.5 mM MgCl₂, 2.5 mM NaHCO₃, 5 mM HEPES, scraped, and solubilized as described above. Heat-stable cytoplasmic extracts were prepared as described previously (33). Briefly, cells were scraped in HBS, immediately heated for 5 min, and centrifugated (20,000 × g for 20 min). Supernatants containing MAPs were diluted 1:1 in 2× sample buffer and heated to 100 °C for 5 min. Brain preparations enriched in MAPs were also obtained from postnatal day 13 and adult rats.

**Western Blotting**—Samples from equivalent number of cells were compared in each experiment. Samples were separated on 8% SDS-polyacrylamide gels. Proteins were transferred electrochemically to polyvinylidene difluoride membranes (Roche Molecular Biochemicals). Membranes were blocked with 1% gelatin, 0.05% Tween 20 in TBS buffer (pH 7.5). After washing with TBS-Tween 20 (0.05%), membranes were incubated overnight at 4 °C with anti-β-tubulin (1:500), anti-IH-tubulin (1:400), tau 1 antibody (1:1000), SE2 antibody (1:1000), or HM-2 antibody (1:500), diluted in TBS-Tween 20 (0.05%) plus bovine serum albumin (0.1%). After washing with TBS-Tween 20, detection was accomplished using horseradish peroxidase-conjugated anti-mouse IgG (1:2000) and an enhanced chemiluminescence detection system. Immunoreactivity was quantified by densitometry with ImageQuant software and expressed as arbitrary units.

**Immunofluorescence Microscopy**—The anti-GFAP antibody was used as glial marker, whereas NE-14 and anti-IIH-tubulin antibodies were used as neuronal markers. Cells were washed twice with HBS, then fixed with methanol for 10 min at −20 °C. Cells were then rehydrated and incubated with anti-IIH-tubulin (1:40), anti-GFAP (1:20), and NE-14 (1:20) antibodies for 1 h at room temperature. After washing, cells were incubated (1 h at room temperature) with an anti-mouse IgG coupled to fluorescein isothiocyanate (1:30). After washing, slides were mounted in Vectashield and examined on a Nikon DM 400 microscope equipped for epifluorescence using a B-1E fluorescein isothiocyanate filter set (Nikon, Melville, NY).

**Measurement of Cell Migration**—To determine cell migration, nuclear DNA staining was performed using propidium iodine as described previously (34) with slight modifications. Cells were washed twice with HBS buffer, fixed with methanol (10 min at −20 °C), rehydrated (for 10 min), and incubated with propidium iodine (1 μg/ml) for 20 min at room temperature. After washing, slides were examined using a G-2A orange range filter set. To quantify cell migration, 12 consecutive rings (multiple of approximately 50 μm), beginning at the edge of the microexplant, were drawn. The percentage of cells that had migrated during the experiment was determined as total number of cells located in a precise ring over the total number of cells from the first to the last ring. The percentage of cells that had migrated was calculated as total number of cells located from the second (first stage of migration) to the last ring, over the total number of cells.

**Data Analysis**—The data are presented as the means ± S.E. Statistical analyses of the data were performed using Student t test, and p values were obtained from Dunnett’s tests. A indicates the number of experiments, each performed in triplicate.

**RESULTS**

As reported previously, cerebellar cells survive and differentiate when cultured as microexplants in conditioned serum-free medium. 24 h after seeding, small clusters of cells exhibited short processes, which increased in number and length as the culture period progressed. After 6 days, cells exhibit several long radially oriented neurites extended from the microplant. Indirect immunofluorescence studies, using neuronal and glial markers indicated that the cell cultures were composed of approximately 50% neurons and 50% glial cells (Fig. 1). Co-incubation of Ang II with AT₁ or AT₂ receptor analogs indicated that the cell cultures contained both AT₁ and AT₂ receptor subtypes (Fig. 2).

Four-day treatment of cerebellar microexplant cultures with 100 nM Ang II induced important morphological changes. Two major modifications were observed. The first involved an increase in length of processes that exhibited several arborizations, and second, a marked cell migration was observed from the edge of the microexplant toward the periphery (Fig. 3, B compared with A). These effects were more pronounced in cells treated with Ang II and DUP 753 (1 μM), a specific AT₂ receptor agonist (Fig. 3C) or in cells treated with 10 nM of CGP 42112, the AT₂ receptor agonist (Fig. 3E). Moreover, incubation with Ang II and PD 123319 (1 μM), a specific antagonist of the AT₂ receptor, blocked the AT₂ receptor-mediated effect (Fig. 3D). Alone, DUP 753 and PD 123319 did not affect cell morphology compared with control cells, whereas PD 123319 abolished the effect of CGP 42112 (data not shown).

Two protocols were used to quantify the morphological changes induced by Ang II treatment. Because process elongation is accompanied by an increase in the polymerization of microtubules, we measured the level of tubulin incorporated into cytoskeletal fractions from control, Ang II-treated, and Ang II-analog-treated cells. As shown in Fig. 4, Ang II alone did not significantly modify the level of polymerized tubulin. However, specific stimulation of the AT₂ receptor through either incubation of CGP 42112 or co-incubation with Ang II and DUP 753
increased the level of polymerized tubulin by 1.67 ± 0.15- and 1.40 ± 0.18-fold, respectively (n = 3). On the other hand, stimulation of the AT1 receptor by co-incubating Ang II with PD 123319 abolished the AT2 receptor-mediating effect (Fig. 4A). However, when incubated alone, DUP 753 or PD 123319 did not modify the basal level of polymerized tubulin, whereas PD 123319 abolished the effect of CGP 42112 (Fig. 4B). By comparison, Ang II and analogs did not change the total level of tubulin content (Fig. 4C). To evaluate whether process elongation affected neurons or glial cells, the effect of Ang II and analogs was veriﬁed on immunofluorescence of βIII-tubulin, an isoform speciﬁcally localized in neurons (35). As shown in Fig. 5, AT2 receptor activation clearly increased the level of βIII-tubulin labeling associated with neurons. After 4 days of treatment with CGP 42112 (Fig. 5E) or Ang II plus DUP 753 (Fig. 5C), the cells had a well developed network of neurites with several varicosities. In these conditions, processes appeared thicker, suggesting that AT2 receptor activation increased fasciculation. Again, morphological observations were correlated with Western blot analyses (Fig. 6) and conﬁrmed that AT2 receptor activation increased the level of βIII-tubulin incorporated into microtubules (Fig. 6, A and B), an effect abolished by the addition of PD 123319 but without any effect on the total level of βIII-tubulin in cells (Fig. 6C).

Synthesis of MAPs represents critical events during elaboration of neurites. Several studies indicate that this synthesis follows a time course that is correlated with axonal and dendritic growth (36). We therefore studied whether Ang II could affect the level of expression of tau and MAP2, two MAPs specifically expressed in axons and dendrites, respectively (36, 37). Tau 1 antibody identiﬁed a group of several tau isoforms. A representative Western blot in Fig. 7A illustrates that AT2 receptor activation by Ang II, after inhibition of the AT1 receptor by DUP 753 or after CGP 42112 treatment, strongly increased the level of the tau protein. In control cells, a single band of 50 kDa was observed, whereas in Ang II plus DUP 753- or in CGP 42112-treated cells, isoforms of higher molecular weight began to appear. The far right lane in Fig. 7A shows the control pattern of tau expression in brain extracts from postnatal day 13 and from adult rats. As expected, a single isoform was detected in the young rat, whereas several bands were revealed in the adult (36–40). Parallel Western blots with the 5E2 antibody, which recognizes unphosphorylated and phosphorylated forms of tau, revealed a stronger effect of Ang II, via the AT1 receptor, suggesting that the difference with the tau 1 immunoblot (Fig. 7A) is due to a preferential increase in tau phosphorylation. Fig. 7 (C and D) illustrates the effect of Ang II and analogs on the level of HMW-MAP2 and on MAP2c. Adult HMW-MAP2 was resolved in two isoforms, termed MAP2a and
MAP2b of 280 kDa; MAP2a is present early in development, whereas the expression of MAP2c disappears in the adult. Again, AT₂ receptor stimulation increased the levels of both proteins. As for tubulin measurements, incubation with DUP 753 or PD 123319 alone exhibited the same immunoreactivity against tau and MAP 2 than control cells (data not shown).

Quantification of cell migration was performed as described under “Experimental Procedures” following cell nuclei labeling with propidium iodine as shown in Fig. 8. Results from Fig. 8A indicate that under CGP 42112 treatment, cells exhibited the highest degree of cell migration. Measurement of the number of migrating cells from the edge of the microexplant to the outermost peripheral ring indicates that Ang II induced a 1.9 ± 0.1-fold increase (n = 3) in the number of migrating cells, compared with control cultures. In corrobororation with microscopic examination, these effects were due to AT₂ receptor activation, because CGP 421122 or Ang II plus DUP 753 induced a stronger effect (3.3 ± 0.21- and 3.1 ± 0.25-fold, respectively, n = 3), whereas co-incubation with PD 123319 blocked these effects (1.2 ± 0.21-fold difference compared with control, n = 3) (Fig. 9).

DISCUSSION

Microexplant cultures of cerebellum were used to study the effect of Ang II on neurite outgrowth as well as on the expression of proteins important for morphological neuronal differentiation. Our results indicate that activation of the AT₂ receptor of Ang II in these Ang II-expressing cells induce important developmental changes, characterized not only by an acceleration of neurite outgrowth but also by cell migration, two features of neuronal maturation of the cerebellum. Moreover, the binding of Ang II to AT₁ receptors antagonizes the effect mediated by the AT₂ receptor. These results are the first to clearly
identify a functional role for Ang II and the AT₂ receptor during the development of a brain structure containing both types of receptors.

Role of Ang II Receptors in Cerebellum Development—Several studies have now demonstrated the presence of AT₁ and AT₂ receptors in the brain (1, 2) and have shown that the AT₂ receptor is mainly expressed during fetal and neonatal brain development (13, 14). In the cerebellum, both types of receptors are present. Autoradiographic studies indicate that AT₁ and AT₂ receptors are found in the molecular layer of the cerebellum of 2-week-old rats but not in the adult, although in situ hybridization revealed that Purkinje cells express AT₁A and AT₁B mRNA but not AT₂ mRNA (4, 25). Using chemical lesion of the inferior olive, Jöhren et al. (26) recently demonstrated that AT₂ receptors were produced in inferior olivary neurons and transported through climbing fibers to the molecular layer of the cerebellar cortex. It should be noted that the concentration of AT₂ receptors found in the inferior olive of young rats (2 weeks old) is severalfold higher than concentrations found in other AT₂-rich areas (3). The present study confirms the hypothesis raised by Jöhren et al. that the high expression of AT₂ receptors in the inferior olive cerebellar pathway may be associated with a role of the AT₂ receptor in neuronal plasticity and cerebellar development. Indeed, as in the neuronal cell line, NG108–15 and in PC12W cells (7, 18), the present study, using cerebellar microexplants in culture, where both neuronal and glial cells are present, indicate that AT₂ receptor activation induces not only neurite outgrowth, a process associated with morphological differentiation but also, interestingly, cell migration.

Pharmacological studies indicate that these effects are abolished by the AT₂ receptor antagonist PD 123319 but increased when Ang II was co-incubated with DUP 753, confirming previous studies that AT₁ and AT₂ receptors have antagonistic actions (8–10, 18). Localization of AT₁ and AT₂ receptors in neurons or glial cells are not yet clearly established. Indeed, binding studies conducted on enriched cultures of astrocytes or neurons from whole brains indicate that AT₁ receptors are predominant in neonatal astrocytes, whereas AT₂ receptors are

FIG. 7. Western blot analysis of the effect of angiotensin II on the level of tau and MAP 2 in cerebellar microexplant cultures. Microexplants were cultured and stimulated for 4 days as a control lane C or with Ang II, Ang II + DUP 753 (+DUP), Ang II + PD 123319 (+PD), or CGP 42112 (CGP) as explained in the legend of Fig. 3. Heat-stable cytoplasmic extracts from experimental microexplants or from postnatal day 13 (P13) or adult rat brain (Adult) were prepared as described under “Experimental Procedures.” Representative Western immunoblotting are shown for the unphosphorylated form of tau, using the tau 1 antibody (A) and for both unphosphorylated and phosphorylated forms, using the 5E2 antibody (B), high molecular isoforms of MAP2 (HMW MAP2) (C), and low molecular isoform, MAP2c (D). Numbers on the left indicate the molecular masses (kDa).

FIG. 8. Effect of Ang II on cell migration in cerebellar microexplant cultures. Microexplants were cultured and stimulated for 4 days without (A) or with Ang II (B), Ang II + DUP 753 (C), Ang II + PD 123319 (D), or CGP 42112 (E) as explained in the legend to Fig. 3. After methanol fixation, cells were processed for immunofluorescence DNA labeling using propidium iodine. The bars represent 50 µm.

FIG. 9. Quantification of the effect of Ang II on cell migration in cerebellar microexplant cultures. A, cell migration was quantified as defined under “Experimental Procedures” by counting the number of cells in a particular ring over the total number of cells from the first to the last ring of migration. Representative analysis for one microexplant is shown. B, quantitative analysis of the percentage of cells that had exhibited migration, calculated as the total number of cells located from the second ring (first stage of migration) to the last ring, over the total number of cells. Results represent the means ± S.E. of three experiments, with a minimum of four microexplants analyzed for each experiment. **, p < 0.001, difference compared with control value.
predominant in neurons (41). However, the latter do express a small proportion of AT2 receptors (approximately 10%). In addition to Ang II receptors, all the components for local production of Ang II production are present in the cerebellum. Angiotensin immunoreactivity has been detected in neurons from Purkinje, granule, basket, and stellate cells (27), as well as renin (42) and angiotensin converting enzyme (43, 44). All these observations support the hypothesis for a specific role of brain Ang II during development. Indeed, the present study indicates that AT2 receptor activation promotes differentiation, characterized here by neurite outgrowth and cell migration, whereas the AT1 receptor inhibits AT2 receptor effects. However, the exact interaction between AT1 and AT2 receptors as well as the interaction between neuronal and glial cells remains to be determined.

Extensive neuronal cell death occurs during cerebellar development, and it is known that AT2 receptors may activate this process (12, 45). However, in our cell culture conditions (microexplants), the most obvious effect of AT2 receptor activation was seen on morphological differentiation plus a newly identified effect on cell migration. Previous studies have effectively shown that cell death was reduced in aggregated cell cultures of cerebellum compared with dissociated cells (46). Because apoptosis versus survival depends on the specific combination of local factors, the effect of Ang II observed in microexplants may be due to interaction of AT2 receptors with factors locally produced by the mixed population of cells present in the microexplants, such as brain-derived neurotrophic factor or neurotrophin 3 (46).

Control of Cell Migration—After their final mitotic division, granule cells actively move through the developing molecular layer to the Purkinje cell layer. This migration is guided by surface-mediated interactions with Bergmann glial fibers that traverse the developing molecular layer (47). However, granular cells lose contact with Bergmann glial fibers after leaving the molecular layer. Furthermore, migration through the internal granular layer is probably controlled by local signaling molecules different from those involved during translocation across the molecular layer (22), from mossy fibers, or from granule cells themselves (48). Pituitary adenyl cyclase activating peptide (49, 50), brain-derived neurotrophic factor and neurotrophin 3 (46) are such factors. Our results indicate that Ang II is also involved in cell migration through the AT2 receptor. As is the case for pituitary adenyl cyclase activating peptide, brain-derived neurotrophic factor, or neurotrophin 3, Ang II is produced in the cerebellum and therefore may act as a local factor (27, 42, 44). On the other hand, Ang II may act directly via regulation of Ca2+ influx. Indeed, it was previously shown that AT2 receptor modulates Ca2+ (51) and K+ channel activities (52), and regulation of Ca2+ influx is an important intracellular mediator controlling cell migration and neurite elongation (53). Alternatively, Ang II may also act indirectly, via the release of local factors, such as plasminogen activator inhibitor 1 (54) or tissue metalloprotease inhibitor (55), the key enzyme for interstitial collagen degradation, both involved in regulating extracellular matrix composition. Knockout mice lacking AT2 receptor have been produced (56, 57). Gross examination of the brain did not reveal changes in brain development, indicating that the other neurotrophic factors could counteract the lack of AT2 receptor or that AT2 receptor was not involved in the organization of the brain itself. However, a decrease in exploratory behavior and locomotor activity was observed, suggesting inappropriate neuronal differentiation in such AT2 knockout mice.

Control of Neurite Outgrowth—Neurite extension is initiated at the growth cone and involves several biochemical steps directed toward promoting of the assembly of tubulin monomers into microtubules necessary to support the growing neurites. The structural subunit of microtubules, tubulin, constitutes a multigene family of isotypes (58). In particular, related to the present work, the class III β-tubulin isoform has a specific neuronal localization (35). Moreover, the level of βIII-tubulin expression is higher in fetal and neonatal brain than in the adults, and its incorporation into neuritic microtubules occurs only after axonal and dendritic differentiation (35, 59). Immunofluorescence studies and Western blot analyses indicate that activation of the AT2 receptor of Ang II induces an increase in the labeling of βIII-tubulin. Thus, the present data indicate that Ang II acts on neurons by increasing elongation of individual neurites, whereas specific activation of the AT2 receptor also increases fasciculation and branching. Of note, βIII-tubulin is the only isoform that can be phosphorylated. This phosphorylation is mediated by casein kinase I and II at a serine residue located in the C terminus or on tyrosine residues (60, 61). These phosphorylations may be important for binding to MAPs, in particular MAP2. Hence, if such mitogen- (or microtubule)-activated protein kinase activation occurs in neuronal cultures βIII-tubulin may be a good candidate as a potential substrate. The differential effect observed with Ang II compared with AT2 receptor alone may be due to positive and negative interactions on protein phosphorylations mediated by the both types of receptors. Indeed, we have shown that AT2 receptor activation induced a slow but sustained increase in mitogen-activated protein kinase activity (62, 63), whereas activation of the AT1 receptor induces a rapid but transient activation of mitogen-activated protein kinase (63).

MAPs play a crucial role to control tubulin polymerization, as well as stability or plasticity of neurite processes (36, 37). Studies by Caceres and Kosik (64) have shown that the initial stage of differentiation, in which cells exhibit exploratory neurites, is dependent on juvenile MAP2c expression. At a later stage, one neurite differentiates into the axon while the others differentiate into dendrites; these changes are coordinated by specific expression of tau in the axon and the adult MAP2 isoform, HMW-MAP2 a and b, termed MAP2. The juvenile forms of tau and MAP2 are highly phosphorylated and are less efficient in promoting microtubule assembly than the adult forms, which are phosphorylated less or not at all. These observations suggest that microtubules in the immature brain are less stable and more plastic than those found in the adult (36, 37). Our results, using different antibodies recognizing unphosphorylated and/or phosphorylated forms of tau as well as MAP2c and the HMW-MAP2, reveal that AT2 receptor stimulation induces changes in tau and MAP2 expression, which follows the same pattern that of βIII-tubulin. Immunofluorescence studies show that the processes induced by Ang II plus DUP 753 and by CGP 42112 are longer and thicker than with Ang II alone, supporting the data that tau and MAP2 functions were stimulated. These findings confirm that AT2 receptor stimulation activates all of the components involved in the process of neurite elongation and that AT2 receptor antagonizes these effects. Several developmental studies indicate that increases in tau and MAP expression precede that of tubulin, suggesting that the primary effect of the AT2 receptor activation may be on tau and MAP2 as such rather than on tubulin itself. Upcoming studies will be performed to detail the time course effect of Ang II on the expression and phosphorylation pattern of these two MAPs. Of note, MAP-2 is the main substrate for ERK1 and ERK2, whereas tau is phosphorylated by several kinases, including proline-directed serine/threonine protein kinase (37), again indicating a possible link with the sustained increase in mitogen-activated protein kinase activity.
we observed previously in NG108–15 cells (62, 63). In conclusion, these results indicate that AT2 receptor activation promotes and/or accelerates all the processes involved in morphological differentiation because its stimulation increases 1) polymerization of the βIII-tubulin, the specific neuronal isoform, 2) tau expression, increasing axonal outgrowth, 3) MAP2c expression, increasing polymerization of microtubules, and 4) HMW-MAP2, increasing stability of mature neurites. Moreover, we identified a new role for the AT2 receptor, which is stimulation of cell migration. These observations clearly demonstrate the involvement of the AT2 receptor in the differentiation of neuronal cells. Both of these functions observed in vitro may occur in vivo, depending on the level of expression and spatial localization of the AT1 and AT2 receptors during development of the cerebellum. In addition, we have shown that the AT2 effects are antagonized by the AT1 receptor, indicating that, as in the control of cellular growth, AT1 and AT2 receptors have opposite actions on neuronal differentiation. With the other environmental growth factors, adhesion molecules, and the components of the extracellular matrix, both receptor types participate in the fine tuning of neuronal differentiation and cell migration, two very important events occurring during cerebellar development. Taken together, the present data indicate that AT2 receptor activation only affects the extent of neurite outgrowth but also affects neurite morphology and as cell migration.

Acknowledgments—We thank Dr. Marcel D Payet and Dr. Christian Casanova for very stimulating discussions and Lucie Chouinard for technical assistance. We are greatly indebted to Dr. Gaetan Guillemette (Department of Pharmacology, University of Sherbrooke, QC, Canada) for the iodination of Ang II, Dr. Kenneth Kosik (Center for Neurologic Diseases, Brigham and Women’s Hospital and Harvard Medical School, Boston, MA) for the gift of the monoclonal tau antibody 5E2, Dr. Lester D. Munsat (Department of Pharmacology, University of Sherbrooke, QC, Canada) for technical assistance. We are greatly indebted to Dr. Gaetan Guillemette (Department of Pharmacology, University of Sherbrooke, QC, Canada) for the gift of CGP 42112. We thank Dr. Marcel D Payet and Dr. Christian Casanova for very stimulating discussions and Lucie Chouinard for technical assistance. We are greatly indebted to Dr. Gaetan Guillemette (Department of Pharmacology, University of Sherbrooke, QC, Canada) for the gift of CGP 42112.

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