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ORIGINAL ARTICLE

In vitro functionality of isolated embryonic hypothalamic vasopressinergic and oxytocinergic neurons: modulatory effects of brain-derived neurotrophic factor and angiotensin II

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Abstract There are only a few studies on the ontogeny and differentiation process of the hypothalamic supraoptic-paraventriculo-neurohypophysial neurosecretory system. In vitro neuron survival improves if cells are of embryonic origin; however, surviving hypothalamic neurons in culture were found to express small and minimal amounts of arginine-vasopressin (AVP) and oxytocin (OT), respectively. The aim of this study was to develop a primary neuronal culture design applicable to the study of magnocellular hypothalamic system functionality. For this purpose, a primary neuronal culture was set up after mechanical dissociation of sterile hypothalamic blocks from 17-day-old Sprague–Dawley rat embryos (E17) of both sexes. Isolated hypothalamic cells were cultured with supplemented (B27)-NeuroBasal medium containing an agent inhibiting non-neuron cell proliferation. The neurosecretory process was characterized by detecting AVP and OT secreted into the medium on different days of culture. Data indicate that spontaneous AVP and OT release occurred in a culture day-dependent fashion, being maximal on day 13 for AVP, and on day 10 for OT. Interestingly, brain-derived neurotrophic factor (BDNF) and Angiotensin II (A II) were able to positively modulate neuropeptide output. Furthermore, on day 17 of culture, non-specific (high-KCl) and specific (Angiotensin II) stimuli were able to significantly ($P < 0.05$) enhance the secretion of both neuropeptides over respective baselines. This study suggests that our experimental design is useful for the study of AVP- and OT-ergic neuron functionality and that BDNF and A II are positive modulators of embryonic hypothalamic cell development.

Keywords AVP · OT · BDNF · A II · Magnocellular neurons

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Introduction

The hypothalamus is a key brain structure integrating information from different brain areas and the periphery. Arginine-vasopressin (AVP) and oxytocin (OT) serve as both neurotransmitters/neuromodulators and peripheral hormones. They are mainly synthesized in the hypothalamic paraventricular (PVN) and supraoptic (SON) nuclei by neurosecretory neurons ranging from magnocellular to parvocellular. Neurons of the PVN and SON transport both AVP and OT to the neurohypophysis where they are stored and later released into the blood stream [1–3]. In final target tissues, these neuropeptides regulate a large number of well-known functions including blood pressure and water metabolism (AVP) [4] and lactation and parturition processes (OT) [1, 5].

Extensive magnocellular neuron death occurs in many organotypic cultures of the neurohypophyseal system. An important factor inductor of this neuron loss is the massive axotomy involved in preparation of hypothalamic tissue for culture [6–8]. This in vitro neuron loss is mainly due to apoptosis [9], and several studies indicate that OT-ergic neurons are more resistant than AVP-ergic neurons for in vitro survival [6, 10–12].

Although some cytokines are able to improve AVP-ergic cell survival in vitro [13], the effect of brain-derived

neurotrophic factor (BDNF) on neuron survival has not been well studied during pre-natal development. BDNF is highly expressed in the hypothalamus [14], even at an early stage of development [15, 16], and high-affinity receptors for BDNF have been detected in hypothalamic neurons [17, 18]. In fact, exogenously added BDNF is able to selectively enhance morphological and functional differentiation of several embryonic neurons in culture, such as dopaminergic [15] and somatostatin-ergic [19]. Angiotensin II (A II) acts at hypothalamic level, stimulating thirst and reducing body water loss. Moreover, A II shows neuropeptide releasing activity upon A II-sensitive hypothalamic neurons, stimulating both AVP and OT output [20]. Although specific A II receptors are already expressed during late embryonic life [21, 22], specific information regarding any in vitro A II effect on embryonic magnocellular neuron survival and function is still lacking.

In this study, and because of the functionally limited ex vivo property of hypothalamic tissue in culture or enzymatically isolated neurons, we examined the in vitro neurosecretory activity of mechanically isolated hypothalamic cells. To achieve this aim, hypthalami from rat embryos processed on gestational day 17 (E17) were used as donors. As an in vitro BDNF-dependent effect on neuron survival was proved to occur when cells were obtained from neonatal rats [23], we also assessed BDNF and A II effects on neuropeptidergic function in our system.

Materials and methods

Animals and tissue

Female Sprague-Dawley rats were mated overnight, and the first day of gestation was determined by the presence of either sperm or a vaginal plug. Hypothalamic blocks were obtained from E17 rat fetuses of both sexes. On the experimental day, dams were lightly anesthetized (ketamine) and sacrificed by decapitation, following protocols for animal use in the NIH Guidelines for care and use of experimental animals. Experiments received approval from our Institutional Committee on Animal Experimentation. E17 brains were rapidly removed in sterile condition, and hypothalami were dissected, with an ophthalmic scissor. Limits were: anterior, the optic chiasm; posterior, borders of mamillary bodies; and lateral, hypothalamic borders; 1 mm depth, approximately [24].

Isolation and culture of embryonic hypothalamic cells

Due to poor in vitro long-term survival of neurons in protocols of hypothalamic slices in culture or enzymatic digestion of these tissues [6–12], in the present work we employed mechanically-isolated hypothalamic cells to test their neurosecretory activity during several days in culture. After careful dissection of hypothalami, tissues were placed in PBS (NaH_2PO_4 1.059 mM-Na_2HPO_4 2.9 mM-NaC1 0.15 M) buffer (pH 7.4), supplemented with 0.06% (w/v) glucose (Carlo Erba, Italy) and antibiotics (100 IU/ml of penicillin and 100 μg/ml streptomycin; Gibco BRL, Life Technologies). Hypothalami were then gently passed several times trough a sterile Pasteur pipette, flamed in the middle to decrease the diameter of their opening. Tissue fragments were allowed to settle, for 4 min, and supernatant was transferred to a clean sterile tube.

Remaining tissue fragments were resuspended in 4 ml of fresh buffer solution, and mechanical dispersion was repeated. Supernatants were then mixed and centrifuged, for 5 min at $100\times g$ at room temperature. The cellular pellet was then gently resuspended in 6 ml of culture medium (Neurobasal Medium, Gibco, containing 2% v/v B27 supplement, Gibco; 500 μM glutamine and 25 μM glutamate, Sigma, Fluka Chemie). To avoid cellular yield and death, the same operator performed the process. Viability of hypothalamic neurons is generally low (30–40% viable cells immediately after the dispersion and before plating, as assessed by Trypan Blue exclusion); a yield of 2×10^5 live cells/hypothalamus was obtained. Cells were then plated, at a density of 190,000 live cells/ml of medium (2.5 ml of cell suspension per well), in 12 well-plates (Corning Costar Corp., Cambridge) containing round coverslips (Marienfeld, Germany), pre-coated with 20 μg/ml poly-D-Lysine (Sigma). Cells grown in culture medium. After 72 h, 2 μM cytosine β-D-arabinofuranoside (β-AraC; Sigma) was added to the culture medium in order to prevent the proliferation of non-neuronal cells. At 72 h post-β-AraC addition, media were replaced by fresh culture medium, although without glutamate to avoid toxicity. Cells were then maintained in culture for a 2 week-period, in sterile condition in a 5% CO_2-85/87% humidity atmosphere, and medium was replaced by fresh medium every two days.

Experimental design and substances tested

The experimental design was drawn as follows. Day 1 of our culture system was considered the day in which medium containing β-AraC was withdrawn and replaced by fresh culture medium. This medium was added either alone or containing one of the following substances: BDNF (Sigma; 1 pM) or A II (Sigma; 100 pM). Media were changed every 2 days, removed media were kept frozen (−20 °C), and then the respective fresh medium was added to the corresponding cell group in culture. On the morning of day 14 of culture, cell functionality was evaluated by removing the media, and replacing them by either fresh

Table 1 Summary of the in vitro culture system for embryonic hypothalamic cells

Day	Action
−2	Culture Medium + β-AraC
1	Culture Medium alone or containing BDNF or A II
14	End of Culture

Between days 1 and 14 media were replaced every 48 h

medium alone (basal) or containing KCl (56 mM) or A II (100 pM). Then cells were cultured in these conditions for 8 h. At the end of culture, media were removed and kept frozen (−20 °C), until measurements AVP and OT concentrations (Table 1).

AVP and OT measurements

Media AVP and OT concentrations were determined by previously reported specific radioimmunoassays [25]. The detection range of the standard curves ranged between 5–500 and 25–1,500 pg/ml, for AVP and OT assays respectively. Coefficients of variation intra- and inter-assay ranged 3–6 and 8–12%, respectively and for both assays.

Statistical analysis

Data were expressed as means ± SEM and were analyzed by multi-factorial ANOVA followed by post-hoc comparisons with Fisher's test [26].

Results

Modulatory effects of BDNF and A II on embryonic hypothalamic cell functionality in a 14-day culture system

Figures 1 and 2 (upper panels) show the results of AVP and OT secretion into the medium on different days of culture. We found no significant amount of either neuropeptide in the incubation medium before day 6 of culture, regardless of medium condition (data not shown).

While spontaneously secreted (cells incubated with Culture Medium only) AVP into the medium was similar on culture days 6 and 8, media AVP concentrations were significantly ($P < 0.05$) increased on culture day 10 (Fig. 1, upper panel). Thereafter, media AVP concentrations returned to day-6 values on days 12 and 14 of culture. Interestingly, the presence of 1 pM BDNF in the medium significantly ($P < 0.05$ vs. respective day-6 values and spontaneous release values on remaining culture days) enhanced AVP medium concentration on day 8 of culture, an effect lasting up to the end of culture (Fig. 1, upper

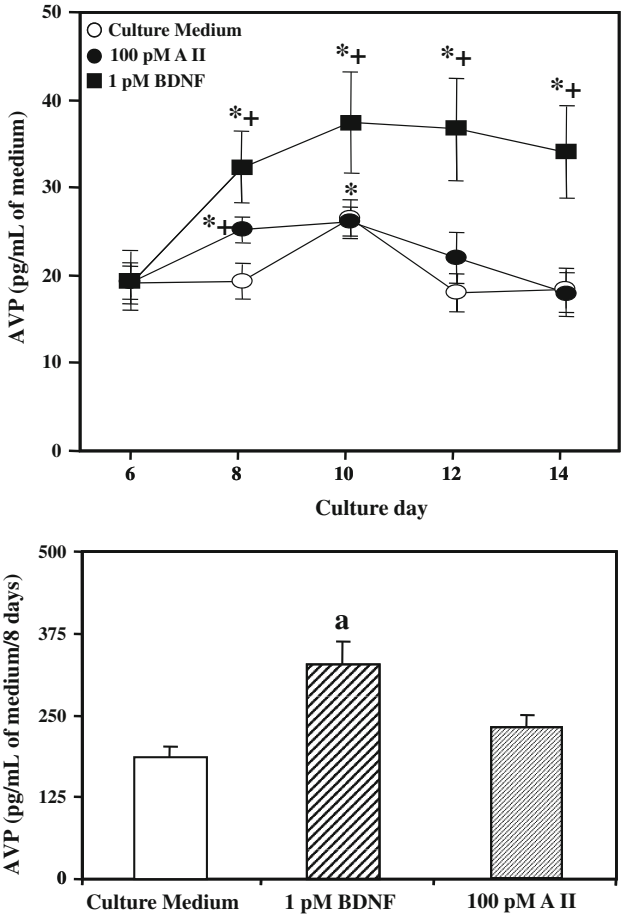

Fig. 1 AVP secretion by isolated hypothalamic neurons on different days of culture in the presence of Culture Medium alone and containing either angiotensin II or brain-derived neurotrophic factor (*upper panel*). The area under the curve of AVP values during an 8-day period of different cell groups in culture is also shown (*lower panel*). Values are mean ± SEM; n = 5/6 experiments, with four wells per condition in each experiment. * $P < 0.05$ versus respective day 6 values. $^{+}$ $P < 0.05$ versus values for Culture Medium on the same day. ^{a} $P < 0.05$ versus Culture Medium and 100 pM A II values

panel). The presence of A II (100 pM) in the Culture Medium significantly ($P < 0.05$ vs. respective day-6 values) enhanced medium AVP concentration on days 8 and 10 of culture (Fig. 1, upper panel). Then values returned to day 6 values on days 12 and 14 of culture (see also Fig. 1, upper panel).

OT secretion into the medium increased spontaneously in a time-related fashion between culture days 6 and 10 (Fig. 2, upper panel). Thereafter, on days 12 and 14 of culture, OT media concentrations returned to values similar to those found on culture day 8. It is noteworthy that when cells were cultured in the presence of 1 pM BDNF, media OT concentrations were similar to those obtained in the presence of Culture Medium only (Fig. 2, upper panel). Conversely, when cells were cultured in the presence of 100 pM A II, a significant ($P < 0.05$ vs. respective day 6-

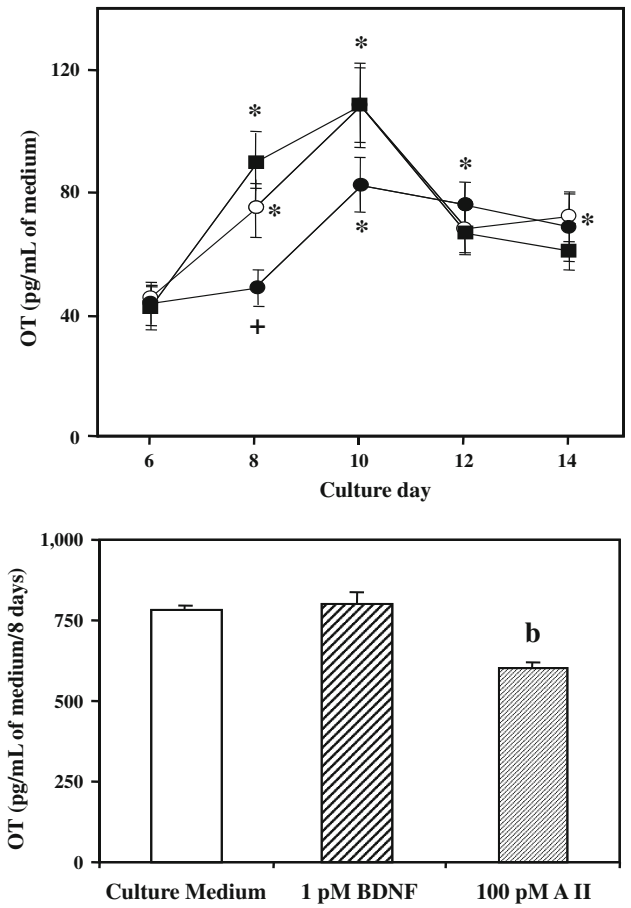

Fig. 2 OT secretion by isolated hypothalamic neurons on different days of culture in the presence of Culture Medium alone and containing either angiotensin II or brain-derived neurotrophic factor (*upper panel*). The area under the curve of OT values during an 8-day period of different cell groups in culture is also shown (*lower panel*). Values are mean ± SEM; n = 5/6 experiments, with four wells per condition in each experiment. * $P < 0.05$ versus respective day 6 values. $^{+}$ $P < 0.05$ versus values for Culture Medium on the same day. ^{b} $P < 0.05$ versus 1 pM BDNF values

values) increment of OT media concentrations was found on culture day 10, a value which held up to the end of the experiment (Fig. 2, upper panel).

Values for the area under the curve (AUC) of AVP and OT media concentrations of different cell groups throughout the entire culture period are shown in Figs. 1 and 2 (lower panels). As depicted, when cells were incubated in the presence of BDNF, the AUC of AVP concentrations was significantly ($P < 0.05$) higher than when cells were exposed to Culture Medium only (Fig. 1, lower panel). Conversely, the AUC of AVP after cells were exposed to A II was similar to values for cells cultured with Culture Medium only. The AUC of OT was similar after exposing cells to either Culture Medium alone or containing A II (Fig. 2, lower panel). Finally, the AUC of OT was significantly ($P < 0.05$) higher in cultures containing BDNF than in those containing A II (Fig. 2, lower panel).

Short-term isolated hypothalamic cell response to unspecific and specific stimuli on day 14 after cultured in different conditions

On the morning of culture day 14, and once media were removed, different cell groups (Culture Medium, containing 100 pM A II or containing 1 pM BDNF) were incubated for 8 h with Culture Medium alone (Basal) or containing 56 mM KCl (hK^{+}). At the end of incubations, we found that all cell groups did respond to hK^{+} stimulation by significantly ($P < 0.05$ vs. respective basal values) enhancing AVP (Fig. 3, upper panel) and OT (Fig. 3, lower panel) secretion into the medium. Important to remark is that basal and hK^{+}-induced AVP secretion was significantly ($P < 0.05$) higher in cells from the BDNF group in cells from the A II-treated group (Fig. 3, upper panel). On the other hand, hK^{+}-elicited OT release was significantly

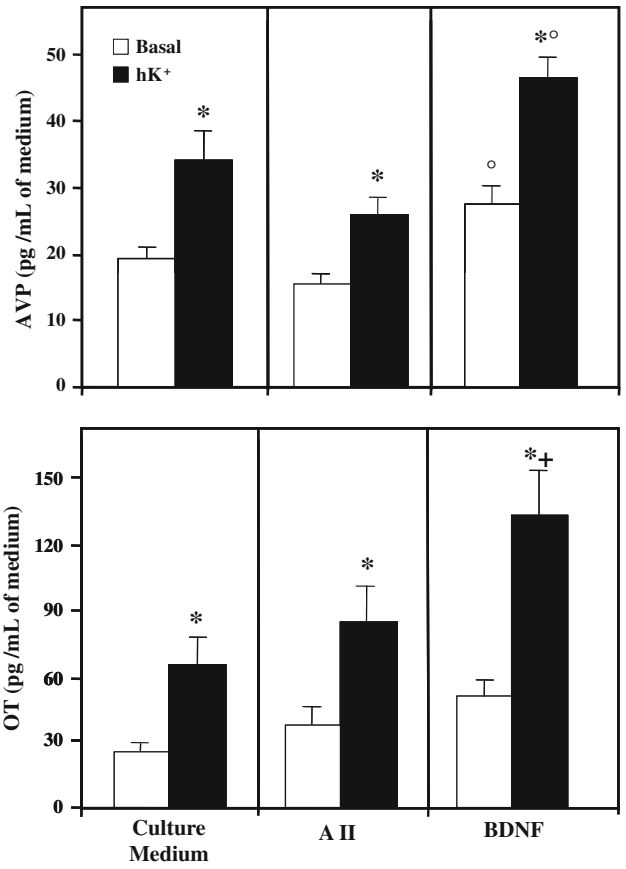

Fig. 3 AVP (upper panel) and OT (*lower panel*) release by isolated hypothalamic neurons (obtained 14 days after culture in the presence of Control Medium only or containing either 100 pM A II or 1 pM BDNF) incubated for 8 h in the presence of Culture Medium alone (Basal) or containing 56 mM KCl (hK^{+}). Values are mean ± SEM; n = 6/8 experiments, with four wells per condition in each experiment. * $P < 0.05$ versus respective Basal values. $^{\circ}$ $P < 0.05$ versus values for the same condition in the A II cell group. $^{+}$ $P < 0.05$ versus hK^{+} values in the Culture Medium cell group

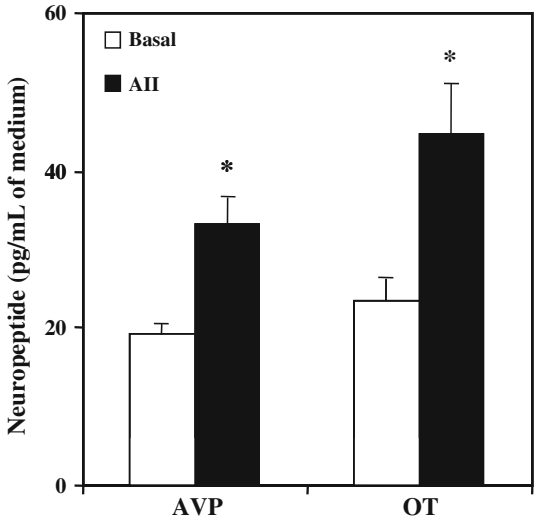

Fig. 4 AVP and OT output by isolated hypothalamic neurons (after 14 days of culture in the presence of Control Medium) incubated for 8 h in the presence of Culture Medium alone (Basal) or containing 100 pM angiotensin II (A II). Values are mean ± SEM; n = 6/8 experiments, with four wells per condition in each experiment. * $P < 0.05$ versus respective Basal values

($P < 0.05$ vs. respective basal values) higher in the BDNF cell group than in the cell group exposed to Culture Medium only (Fig. 3, lower panel).

Finally, cells incubated for 14 days with Culture Medium only were also exposed for 8 h to either Culture Medium alone (Basal) or containing 100 pM A II. Results (Fig. 4) indicate that these cells did respond to A II by significantly ($P < 0.05$) enhancing AVP and OT release over respective basal values.

Discussion

Our study demonstrates that isolated E17 hypothalamic cells from rats of both sexes survived in vitro for a total period of 17 days (3 and 14 days in the presence and absence of β-AraC, respectively). Our study also demonstrated that specific secretory activity (in terms of both AVP and OT release) became measurable on day 6 of culture and held up to the last experimental day, even when exposed to Culture Medium alone. It is worth noting that while a specific stimulus for AVP- and OT-secreting neurons such as A II somewhat facilitated neuronal spontaneous AVP but not OT secretion, specifically, between days 6 and 10 of the culture system. Interestingly, when BDNF was present in culture a positive effect on AVP secretion also developed, although the effect of BDNF on neuron AVP secretion occurred in a more robust fashion and lasted up to the end of the culture system. Unlike AVP, the presence of A II in the culture system somewhat reduced OT secretion (vs. spontaneous output) between days 8 and 10 of culture. However, BDNF had no effect on OT secretion by hypothalamic cells in culture. It is relevant that after 14 days of culture peptidergic cells did display a significant acute neurosecretory function, regardless of previous culture condition. In fact, they were able to develop a significant response to a non-specific (hK +) stimulus. Cells cultured for 14 days with medium alone were also able to develop a secreting response 8 h after incubation with a specific stimulus (A II).

Data on neuron neuropeptide secretion in our system suggest that BDNF could act as a differential modulator of hypothalamic magnocellular neuron maturation and functionality, since BDNF added to the medium enhanced AVP-ergic activity whereas OT-ergic function remained the same, thus supporting that BDNF could be selectively modulating in vitro the functionality of one population of isolated SON–PVN neurons. As BDNF is highly expressed in the developing rat hypothalamus [27, 28], as is its specific receptor TrkB [17], it is plausible to expect an autocrine/paracrine role for BDNP during hypothalamic neuron development. In fact, BDNF has been shown to increase in vitro hypothalamic dopaminergic neuron survival [29]. Also supporting differential effects of BDNF on distinct neuron populations, BDNF has been claimed to enhance dopaminergic neuron differentiation when cells were isolated from the periventricular nucleus but not if collected from the arcuate nucleus [15]. However, we cannot ascertain throughout our study the nature of the receptor involved in the BDNF effect on AVP-releasing cells, either specific high-affinity TrkB receptor or low-affinity neurotrophin receptor p^{75NRT} or both [17, 18].

The in vitro design employed in this study is relevant because of the maintenance of clear functionality of isolated hypothalamic neurons over in vitro development even after several days in culture. It is known that while hypothalamic organotypic explants in culture are able to retain cytoarchitectonic organization and topographic relationships [30], survival of AVP and OT neuron activities significantly declines during the preparation of explants due to apoptosis [6–8, 30]. This characteristic could be analogous to some data observed after in vivo manipulations in rats, specifically for AVP and OT neuron populations [31]. Interestingly, it has been shown that neurotrophic factors such as ciliary neurotrophic factor (CNTF) and leukemia inhibiting factor [7, 30] and apoptosis inhibitors [9] are able to protect magnocellular neurons against programmed apoptosis in cultured hypothalamic explants; however, whereas it was shown that early presence of CNTF is required [32], conversely, BDNF was unable to exert such an effect [7]. In this study we were able to address a positive modulatory effect of BDNF on isolated AVP-ergic functionality. Our findings found strong support in an elegant study by Aliaga et al. [33], clearly addressing

autocrine/paracrine effect of BDNF on AVP-ergic neuron function. They found that osmotic stress enhanced hypothalamic BDNF mRNA expression preceding that of AVP mRNA in the SON. Similarly, other studies indicate that PVN AVP neuron function is regulated by exogenously administered BDNF [34]. In our isolated embryonic neuron system, a clear effect of A II on both neuropeptides secretion was found, thus indicating a clear neurotrophic effect. This event is strongly supported by studies indicating that specific A II receptors (types 1 and 2) are already present in brain areas of embryos from late gestating rats [21, 22]. The A II dipsogenic effect takes place to maintain water homeostasis by activating neurons present in several brainstem sites including the PVN–SON [35]. Although our study does not allow us to ascertain whether the neurotrophic effect of A II on AVP- and OT-secreting cells was due to its binding to specific A II receptors or not [36], we have now shown that the secretory mechanism [20] is fully active when our cells were exposed to A II stimulation, suggesting a positive role in embryonic hypothalamic neuron development.

In summary, our study strongly supports that mechanically isolated hypothalamic neurons from E17 rats of both sexes are able to survive in vitro for several days. Importantly, the cell population retained a functional capacity even up to 17 days after isolation. We also assessed in vitro modulatory effects of BDNF and A II on isolated neuropeptidergic cell survival.

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