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Potent activation of FGF-2 IRES-dependent mechanism of translation during brain development

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ABSTRACT

Fibroblast growth factor-2 (FGF-2) plays a fundamental role in brain functions. This role may be partly achieved through the control of its expression at the translational level via an internal ribosome entry site (IRES)-dependent mechanism. Transgenic mice expressing a bicistronic mRNA allowed us to study *in vivo* and *ex vivo* where this translational mechanism operates. Along brain development, we identified a stringent spatiotemporal regulation of FGF-2 IRES activity showing a peak at post-natal day 7 in most brain regions, which is concomitant with neuronal maturation. At adult age, this activity remained relatively high in forebrain regions. By the enrichment of this activity in forebrain synaptoneuroosomes and by the use of primary cultures of cortical neurons or cocultures with astrocytes, we showed that this activity is indeed localized in neurons, is dependent on their maturation, and correlates with endogenous FGF-2 protein expression. In addition, this activity was regulated by astrocyte factors, including FGF-2, and spontaneous electrical activity. Thus, neuronal IRES-driven translation of the FGF-2 mRNA may be involved in synapse formation and maturation.

Keywords: FGF-2; protein synthesis; IRES; development; brain; neuron

INTRODUCTION

During their whole life span, cells from the central nervous system (CNS) are subjected to plastic changes, which depend on the action of growth factors like fibroblast growth factor-2 (FGF-2). Indeed, FGF-2 plays multiple roles both during CNS development and in the adult, such as in proliferation and differentiation of neural precursor cells (neurons or glia), neuritogenesis, synapse formation, neuroprotection, cognitive processes, post-lesional repair, and blood–brain barrier formation (Vaccharino et al. 1999; Gremo and Presta 2000; Reuss and von Bohlen und Halbach 2003). This prominent role is illustrated by the FGF-2 knock-out mice that coped with severe neuronal and glial

deficits particularly in the cerebral cortex (Dono et al. 1998; Ortega et al. 1998; Vaccharino et al. 1999; Korada et al. 2002).

The wide range of FGF-2 cerebral functions may be partly achieved by the control of its expression at the translational level. For instance, the FGF-2 mRNA contains multiple translational alternative initiation sites leading to the synthesis of five isoforms (Florkiewicz and Sommer 1989; Prats et al. 1989; Arnaud et al. 1999; Touriol et al. 2003). In addition, this mRNA exhibits an internal ribosome entry site (IRES) in its 5′ untranslated region (UTR) (Vagner et al. 1995). Consequently, it can be alternately translated either by the classical cap-dependent mechanism or by the unusual IRES-dependent mechanism. Such a peculiar mechanism of translation was first discovered for picornavirus mRNAs, which lack a cap in their 5′ UTR (Pelletier and Sonenberg 1988), and subsequently characterized in eukaryotic mRNAs, first in Bip mRNA (Macejak and Sarnow 1991) and then in a growing number of mRNAs (van der Velden and Thomas 1999; Bonnal et al. 2003; Stoneley and Willis 2004; Komar and Hatzoglou 2005; Baird et al. 2006).

The cellular events driving IRES-dependent translation are, however, still far from being understood. IRESs may

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allow translation in a proper time and space when cap-dependent translation is impaired. Accordingly, cellular stress or mitosis, which leads to inhibition of total protein synthesis through the blockade of cap-dependent translation, favors IRES-dependent translation (Bonnal et al. 2003; Qin and Sarnow 2004; Sherrill et al. 2004; Holcik and Sonenberg 2005). Using transgenic mice expressing a bicistronic mRNA, Creancier et al. (2000) found a heterogeneous distribution of this FGF-2 IRES activity throughout adult tissues, and notably a high IRES activity in the brain. In the adult, FGF-2 is localized in neurons as well as in astrocytes and implicated in brain plasticity (Ishiyama et al. 1991; Abe et al. 1992), a phenomenon that recapitulates some developmental events. Accordingly, we decided to determine whether FGF-2 IRES-dependent translation could be part of these processes, by studying where and when this translational mechanism operates in the CNS.

In this article, we reveal a specific and stringent spatio-temporal regulation of FGF-2 IRES activity throughout brain regions. At the cellular level, a strong IRES activity was detected in a neuronal compartment, the synaptoneuro-somes. Studies carried out on primary cultures or cocultures of cortical neurons and astrocytes confirmed the neuronal localization of this activity and revealed its dependency on neuronal maturation and its regulation by astrocyte factors as well as by electrical activity.

RESULTS

Bicistronic transgenic mouse model

The bicistronic transgenic mouse represents an appropriate model to study the regulation of FGF-2 IRES-dependent translation in an *in vivo* context. The bicistronic construct expressed under the control of the cytomegalovirus promoter contains an upstream cistron coding for *Renilla* luciferase (RLuc), which is separated from a downstream cistron coding for *Firefly* luciferase (FLuc) by the sequence of the FGF-2 IRES (Fig. 1A; Creancier et al. 2000). Enzymatic activity of RLuc reflects the levels of cap-dependent translation and is directly correlated with the number of bicistronic transcripts. On the other hand, enzymatic activity of FLuc corresponds to the levels of FGF-2 IRES-dependent translation. Consequently, the ratio of FLuc to RLuc activity reflects FGF-2 IRES activity, independently of the transgene expression level.

Regional distribution of FGF-2 IRES activity in the adult mouse brain

In the adult (P42), FGF-2 IRES activity measured in various brain regions of bicistronic transgenic mice was heterogeneous, being particularly high (ratio above 10) in neocortex (CTX) and striatum (STR) (Fig. 1B; Table 1). A lower but still consistent activity was observed in the hippocampus

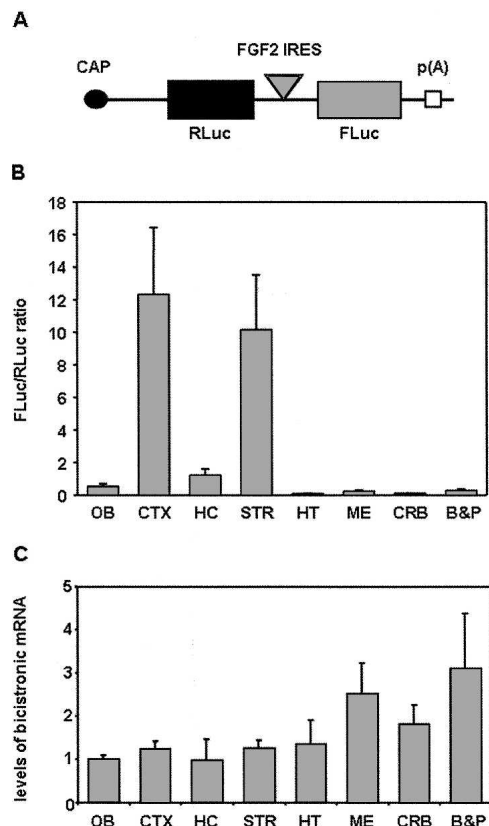


FIGURE 1. Brain distribution of FGF-2 IRES activity in the adult transgenic mouse. (A) Schematic representation of the bicistronic luciferase mRNA construct containing the FGF-2 IRES. The plasmid (pCRFL) used to generate RFL12 transgenic mice is described in Creancier et al. (2000). Expression of the transgene is under the control of the CMV promoter. (B) Regional distribution of FGF-2 IRES activity. Homogenates were prepared from different brain areas of adult RFL12 transgenic mice. (OB) olfactory bulb, (CTX) neocortex, (HC) hippocampus, (STR) striatum, (HT) hypothalamus, (ME) mesencephalon, (CRB) cerebellum, (B&P) bulb and pons). *Renilla* (RLuc) and *Firefly* (FLuc) luciferase activities were measured as described in Materials and Methods. FGF-2 IRES activity is calculated by the ratio FLuc/RLuc. Values represent the means \pm SEM of determinations obtained from nine mice. (C) Regional distribution of the bicistronic mRNA. RNAs were extracted from the same brain areas and reverse transcribed. Levels of bicistronic mRNAs were measured by real time RT-PCR as described in Materials and Methods. Expression levels in OB (Ct values = 30.4 ± 0.6) were set to 1 as the calibrator and the values are given as mean ratios \pm SEM of bicistronic mRNAs in one brain area relative to those measured in OB. These values were obtained in four separate experiments.

(HC) and olfactory bulb (OB) (ratio around 1 and 0.5, respectively). In other cerebral regions, hypothalamus (HT), cerebellum (CRB), mesencephalon (ME), and bulb and pons (B&P), ratios of activity ranged from 0.08 to 0.28, but these lower values were still higher than those previously measured in other organs by Creancier et al. (2000). Similar values were obtained in the brain regions of heterozygote mice and of another strain of bicistronic transgenic mice, RFLD (data not shown). Expression levels of bicistronic mRNAs measured by quantitative RT-PCR were rather

TABLE 1. FLuc and RLuc activities in brain areas during development

	E16	E19	P1	P3	P7	P14	P21	P42
B	958 ± 69	2141 ± 227	2987 ± 360	4046 ± 1150	7255 ± 1549	2957 ± 489	932 ± 127	1083 ± 174
	5569 ± 1234	69,180 ± 13,085	105,589 ± 17,160	82,006 ± 9901	7578 ± 1221	3148 ± 477	1297 ± 155	2440 ± 526
CTX	1129 ± 258	2711 ± 109	32,075 ± 5711	71,344 ± 9682	29,4605 ± 18,661	71,796 ± 8454	34,404 ± 3193	18,620 ± 846
	3407 ± 779	3535 ± 101	22,260 ± 2704	13,074 ± 3658	4249 ± 528	3232 ± 260	1564 ± 94	1730 ± 269
HC	1212 ± 265	376 ± 38	5365 ± 564	6078 ± 454	19,515 ± 1852	6870 ± 913	2330 ± 251	2166 ± 257
	6799 ± 1021	12,308 ± 5801	51,454 ± 9867	15,032 ± 2517	1922 ± 160	2478 ± 429	1342 ± 42	1817 ± 205
STR	1001 ± 122	764 ± 90	4600 ± 1306	2214 ± 545	85,592 ± 15,505	44,661 ± 8406	17,495 ± 2814	9077 ± 1162
	1958 ± 402	1559 ± 131	11,421 ± 4314	3860 ± 996	2282 ± 208	2587 ± 330	885 ± 135	969 ± 154
HT	355 ± 69	474 ± 44	2387 ± 300	2688 ± 240	3599 ± 556	851 ± 55	167 ± 18	165 ± 21
	3308 ± 702	2538 ± 587	12,133 ± 2566	5894 ± 1482	1391 ± 178	2040 ± 519	1318 ± 135	2466 ± 393
ME	n.d.	n.d.	15,245 ± 1599	9162 ± 381	7546 ± 929	1108 ± 90	823 ± 185	716 ± 94
	n.d.	n.d.	21,073 ± 2781	4172 ± 453	1691 ± 165	2526 ± 281	3602 ± 675	3277 ± 729
CRB	n.d.	n.d.	17,590 ± 2100	14,436 ± 752	8530 ± 516	466 ± 97	234 ± 37	137 ± 13
	n.d.	n.d.	61,824 ± 16,429	16,777 ± 514	6259 ± 313	1711 ± 354	1057 ± 129	1892 ± 356
B&P	4457 ± 908	7691 ± 333	19,072 ± 2856	16,283 ± 1240	14,515 ± 2157	2432 ± 664	507 ± 118	817 ± 57
	20,452 ± 4542	3777 ± 482	14,678 ± 3193	6146 ± 701	1842 ± 220	2761 ± 597	3075 ± 130	3348 ± 418

FLuc (upper italic values) and RLuc (lower plain text values) activities were measured in proteins extracts from several brain structures from embryonic E16 to postnatal P42 transgenic mice. Data are given in arbitrary units of luciferase activity/mg tissue and represent means ± SEM of the raw values (from four to nine mice) that were used to calculate the FLuc/RLuc ratios presented in Figures 1, 2, and 3. (n.d.) not determined.

homogeneous throughout all brain areas, except in ME and B&P (Fig. 1C). Interestingly, the higher mRNA expression in these two brain areas was correlated with a higher RLuc activity (Table 1).

Altogether, these findings reveal that the different levels of FLuc activity observed in various brain areas are not linked to variations in bicistronic mRNA transcription, and thus probably result from a differential activation of the FGF-2 IRES-dependent translation.

Developmental regulation of FGF-2 IRES activity in various brain structures

As FGF-2 is implicated in brain development, we measured FGF-2 IRES activities from embryo (E16) to adulthood (P42) in different brain structures (Fig. 2). Before E16, the IRES activity determined in body and head of the embryo at E11 was extremely low, i.e., 0.060 ± 0.001 (FLuc/mg tissue = 1228 ± 219 ; RLuc/mg tissue = $19,968 \pm 4196$) and 0.087 ± 0.003 (FLuc/mg tissue = 303 ± 17 ; RLuc/mg tissue = 3481 ± 116), respectively.

In all brain areas except OB, a peak of high FGF-2 IRES activity was observed at P7, although with different patterns from one area to another (Fig. 2; Table 1). In the OB, the IRES activity after P7 reached a plateau and decreased only slightly in the adult. In CTX, STR, and HC, a sharp increase was observed between P3 and P7, followed by a decrease between P7 and P14, and finally a plateau. In contrast to these regions, the IRES activity in CRB, HT, ME, and B&P decreased after P7 and rapidly dropped to the low values observed in the adult. The B&P was the only structure, which showed a substantial IRES activity (above 2) earlier, in the E19 embryo. It should be mentioned that a peak of RLuc activity took place at P1 in all brain areas, the following decrease being concomitant with the increase in FLuc activity, which peaked at P7.

Taken together, this developmental study reveals the existence of a stringent spatiotemporal regulation of FGF-2 IRES activity from embryonic life to adulthood.

FGF-2 IRES activity throughout cortical areas

As illustrated in Table 2, we analyzed more precisely the distribution of IRES activity within the highly structured neocortex and found marked differences between each subcortical area. The highest IRES activities were detected both in frontal and temporal CTX, two regions known to be involved in long-term memory storage. About 3 times lower IRES activities were observed in parietal and cingular CTX, and the lowest activity was found in the occipital visual region. The heterogeneity of IRES activity within the CTX strongly supports the participation of this mechanism of translation in specific brain functions of FGF-2.

Specificity of the FGF-2 IRES activity in brain regions at P7

As FGF-2 IRES activities were very high at P7 in most brain regions, we investigated whether this peak of activity could be observed in other tissues. In kidney and heart, FLuc/RLuc ratios were very low (0.020 ± 0.001 and 0.011 ± 0.001 , respectively) at P7 and similar to those found in the adult (Creancier et al. 2000). Accordingly, the high FGF-2 IRES activity and its peak at P7 are specific to the cerebral tissue, and its temporal variation suggests a link with brain-specific developmental processes.

The specificity of FGF-2 IRES activity at P7 was further addressed by the comparison with other IRESs in similar bicistronic transgenic mice (Fig. 3). IRES activities of vascular endothelial growth factor (VEGF), of the proto-oncogene c-myc, and of the encephalomyocarditis virus (EMCV) were much lower than that of FGF-2 in all brain areas at P7: FLuc/RLuc ratios ranged from 0.2 to 0.8 for c-myc and EMCV IRESs, and those for VEGF were even lower (around 0.06). In addition, only small variations were detected from one brain area to another. All these data emphasize the specific regulation pattern of the FGF-2 IRES element.

Integrity of the bicistronic mRNA

We then verified that the specific increase in IRES activity observed at P7 did not result from cap-dependent translation of monocistronic Fluc mRNAs (Han and Zhang 2002; Kozak 2003; Van Eden et al. 2004; Liu et al. 2005; Wang et al. 2005). This possibility was checked by the sensitive quantitative RT-PCR method using RLuc- and FLuc-specific probes for the detection of bicistronic and putative additional monocistronic transcripts (Teshima-Kondo et al. 2004; Holcik et al. 2005). In RNAs extracted from both P7 forebrain and adult brain of transgenic mice, the equimolar concentration of both cistron transcripts (Fig. 4) showed the integrity of the bicistronic mRNA, thereby confirming that the stringent regulation observed during brain development results from the specific activation of the IRES-dependent mechanism of translation (Creancier et al. 2000; Teshima-Kondo et al. 2004).

Neuronal localization of FGF-2 IRES activity

As FGF-2 mRNA and proteins have been localized in both neuronal and glial cells (Gomez-Pinilla et al. 1994), we investigated whether FGF-2 IRES activity was present in both cell types. First, we measured the IRES activity in a fraction enriched in synaptoneuroosomes from P6–P7 transgenic mouse forebrain. The quality of the enrichment was revealed by the concomitant enhancement in a synaptic terminal marker (post-synaptic density PSD95 protein) content and impoverishment in a glial marker (glial fibrillary acidic protein [GFAP]) content within the preparation (Fig. 5A). Interestingly, the FLuc/RLuc ratio reached 38

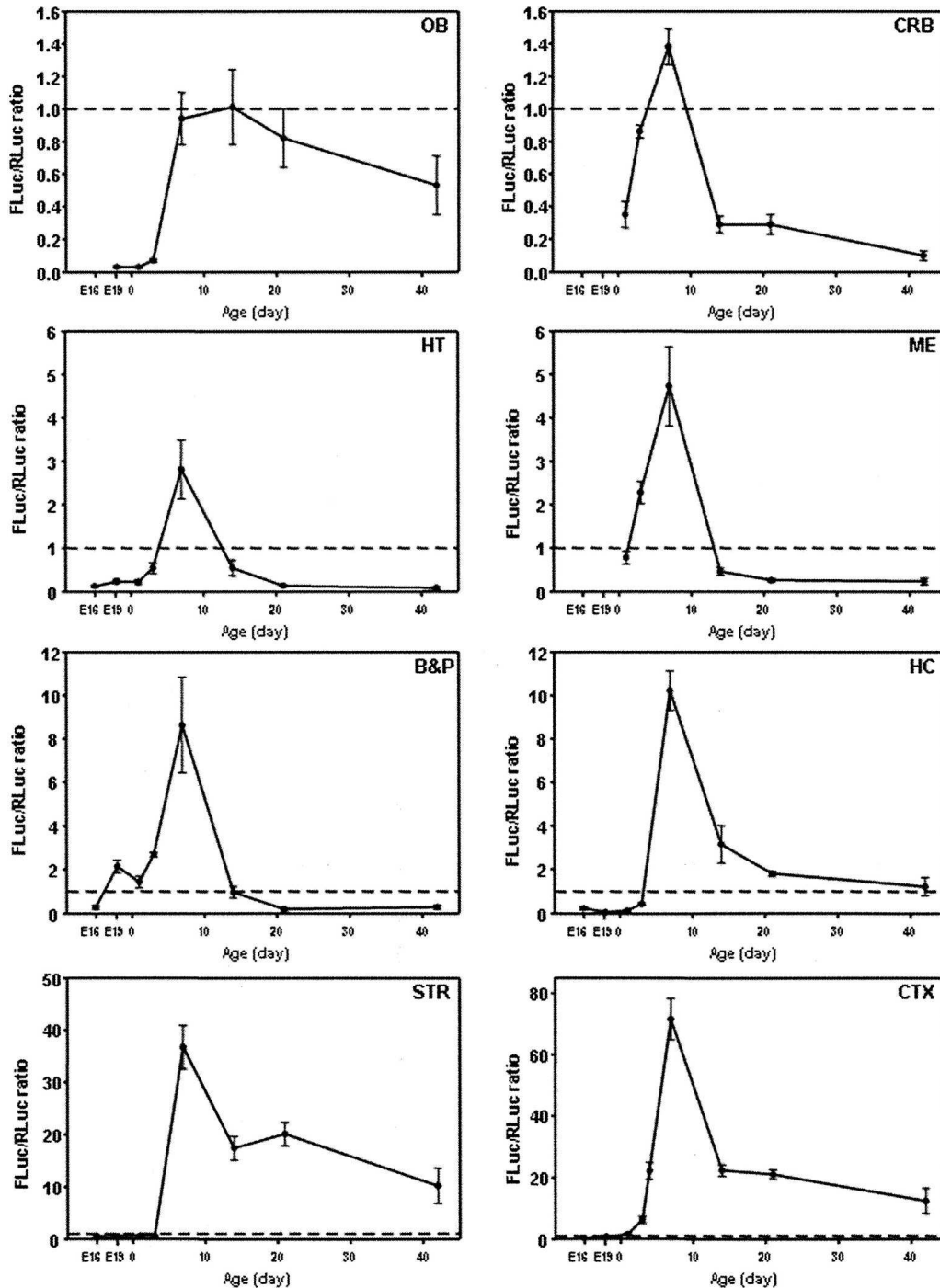


FIGURE 2. Spatiotemporal regulation of FGF-2 IRES activity during embryonic and postnatal development of transgenic mice. Homogenates were prepared from different brain areas of E16 to P42 RFL12 transgenic mice. Luciferase activities were measured as described in Materials and Methods. FGF-2 IRES activity is given as the FLuc/RLuc ratio and the dotted line corresponds to a ratio of 1. Values represent the means \pm SEM of determinations obtained from four to nine mice.

in the synaptoneurosoma fraction, i.e., twice the ratio measured before enrichment (Fig. 5B).

We then measured FGF-2 IRES activity in primary cultures of cortical neurons or astrocytes from transgenic mice. It is noteworthy that FGF-2 IRES activities in freshly

dissociated cortical cells were similar to those measured in whole intact CTX at the same developmental stage, i.e., very low at E16 (ratio of 0.03) and much higher at P3–5 (ratio above 10) (Table 3). After 10 d in vitro (DIV), FLuc/RLuc ratio was above 1 in neurons and very low (0.05) in

TABLE 2. Heterogeneity of FGF-2 IRES activity in the mouse neocortex

Cortical area	FGF-2 IRES activity (% of whole neocortex)
Frontal	160 ± 24
Parietal	59 ± 5
Temporal	129 ± 12
Occipital	31 ± 5
Cingular	48 ± 19

Neocortex from P7 RFL12 mice was subdivided and FGF-2 IRES activity measured, as described in Materials and Methods. Data are expressed as percentages of the values obtained in the whole CTX of transgenic mice. Values represent the means ± SEM of measurements in four mice.

astrocytes, corresponding to a 49-fold increase in activity for neurons and a 289-fold decrease for astrocytes.

Finally we visualized *in situ* the cellular localization of the Firefly reporter protein by immunocytochemistry in 10 DIV neuronal cultures, where less than 10% astrocytes can be observed. Neurons identified by specific markers, β -tubulin or microtubule associated protein 2 (MAP2) (red labeling), displayed a strong FLuc green immunofluorescent signal (Fig. 5C, i,ii), which was present in most neurons (Fig. 6D). On the other hand, FLuc signal was never detected in astrocytes, easily identified by the GFAP immunoreactivity (red labeling) (Fig. 5C, iii). A typical astrocyte surrounded by neuronal projections and devoid of FLuc can also be observed in Figure 5C, ii.

Taken together, these data demonstrate that the FGF-2 IRES-dependent translation takes place in neurons.

Regulation of FGF-2 IRES activity in cortical cultures of neurons and cocultures of neurons/astrocytes

The increase in IRES activity during the first post-natal week coincides with a period of intense astrocyte proliferation, and these cells are known to strongly influence neuronal development both *in vivo* and *in vitro* (Lieth et al. 1989; Hertz 1991; Goritz et al. 2002; Lippman and Dunaevsky 2005). We therefore examined the regulation of IRES activity both in cultures of neurons and in cocultures of neurons/astrocytes.

Anti- β -tubulin immunocytochemistry clearly revealed that the cocultures displayed both at 3 DIV and 10 DIV a large increase in neuritic processes when compared to the neuronal cultures (Fig. 6A). From 3 to 19 DIV, we observed in these cocultures a stringent regulation of IRES activity (Fig. 6B), with a remarkable peak at 10 DIV. Interestingly, this activity at 3 DIV was already 10 times higher (0.3) than in the E16 dissociated cells (0.03), increased at each step until 10 DIV, where it reached the highest value (FLuc/RLuc ratio around 10), and subsequently dropped. Although neuronal cultures presented similar kinetics of IRES

activity with the peak at 10 DIV, changes of IRES activity occurred later, after 5 DIV, and levels of activity were much smaller at any developmental stage (Fig. 6B). The transient peak of IRES activity resulted from opposite variations of FLuc and RLuc activities in the cocultures (Fig. 6C) and in neuronal cultures (data not shown). The highest RLuc activity was observed at 3 DIV while the highest FLuc activity was detected at 10 DIV, corresponding from 3 DIV to 10 DIV to a sevenfold decrease in RLuc activity and a

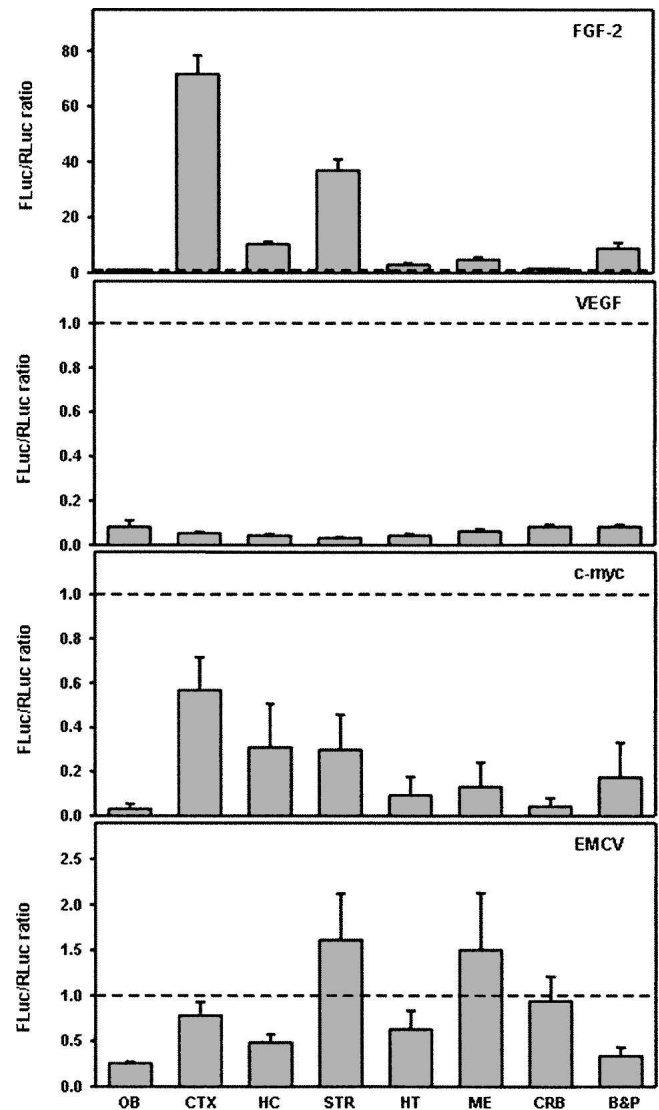


FIGURE 3. Brain distribution of different IRES activities in P7 transgenic mice. Transgenic mice expressing bicistronic constructs bearing IRES from either eukaryotic (FGF-2, VEGF, and VEGF) or picornaviral (EMCV) mRNA are described in Materials and Methods. Homogenates were prepared at postnatal day P7 from various brain areas of the different transgenic mice. Luciferase activities were measured as described in Materials and Methods. FGF-2 IRES activity is given as the FLuc/RLuc ratio, and the dotted line corresponds to a ratio of 1. Values represent the means ± SEM of determinations obtained from four to nine mice.

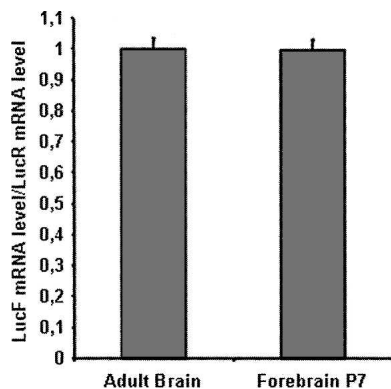


FIGURE 4. Bicistronic mRNA integrity. RNAs were prepared from P7 forebrains and adult brains of RFL12 transgenic mice and reverse transcribed as described in Materials and Methods. Levels of reporter RLuc and FLuc mRNAs were measured by a real-time RT-PCR as described in Materials and Methods. The Ct values obtained with RLuc probes were 28.56 ± 0.1 in the adult and 29.51 ± 0.06 at P7 whereas the Ct values obtained with Fluc probes were 28.78 ± 0.1 in the adult and 29.49 ± 0.05 at P7. Results are expressed as the mean ratio of FLuc relative mRNA level/RLuc relative mRNA level \pm SEM resulting from triplicate determinations obtained in two mice.

fourfold increase in FLuc activity. To determine whether changes of bicistronic mRNA expression might be involved in these opposite variations, levels of these mRNAs were measured by real time RT-PCR at these two representative developmental stages of cell cultures. As the Ct values obtained at 3 DIV ($Ct = 33.7 \pm 1.8$, $n = 6$) and 10 DIV ($Ct = 33.4 \pm 0.5$, $n = 6$) were not significantly different, it strongly indicated that the variations in luciferase activities do not result from changes of bicistronic mRNA expression, but rather correspond to a switch in translational mechanism. In addition, the change in FLuc activity was reflected by a similar variation in the immunofluorescence FLuc signal intensity detected in these neuronal cultures (Fig. 6D).

In order to address the physiological relevance of these findings, we analyzed the endogenous expression of the FGF-2 protein at the same developmental stages in neuronal cultures. While absent at 3 DIV, FGF-2 protein (red labeling) was detected into neurons at 10 DIV, mostly as a cytoplasmic punctuated signal (Fig. 6E).

In these *ex vivo* studies, we characterized a sharp regulation of FGF-2 IRES-dependent translation in neurons, which correlates with the temporal expression of endogenous FGF-2 and is strongly potentiated by astrocytes.

Regulation of FGF-2 IRES activity by astrocyte conditioned medium and growth factors

An important role of astrocytes is to actively participate in neuronal maturation, partly through diffusible signals. Interestingly, addition of astrocyte-conditioned medium (ACM) to cortical neurons induced a strong increase in IRES activity at 3 DIV (Fig. 7A), which reached a value

(0.22) close to that obtained in neuron/astrocyte coculture (0.27), indicating that diffusible factors released from astrocytes stimulate the neuronal FGF-2 IRES activity. This effect is transient; indeed, the ACM was no longer effective at 5 DIV (data not shown).

In order to identify some of these diffusible factors, we investigated the potential involvement of FGF-2 itself, as it is released by astrocytes and favors neuronal survival and development (Walicke et al. 1986; Unsicker et al. 1987; Mattson and Rychlik 1990). Inhibition of its receptor by the antagonist SU5402 (Mohammadi et al. 1997) significantly decreased (about 30%) the effect of ACM on FGF-2 IRES activity (Fig. 7A). In addition, FGF-2 treatment increased the FLuc/RLuc ratio in a dose-dependent manner (Fig. 7B), and the maximal increase obtained with 10 ng/mL FGF-2 suggested the involvement of high affinity FGF-2 receptors. This increase was similar to that induced by the ACM treatment (Fig. 7A) or

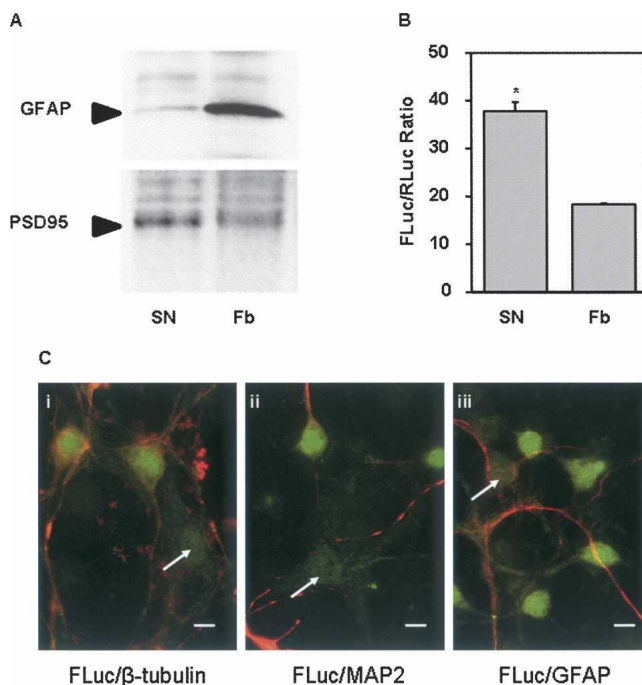


FIGURE 5. Cellular localization of FGF-2 IRES activity. (A) Western blots were performed on protein extracts (Fb) or a synaptoneurosomal preparation (SN) from forebrains of P6–7 transgenic mice. Immunoblotting was performed with antibodies raised against either the GFAP astrocyte marker or the PSD95 nerve terminal marker, respectively. (B) FGF-2 IRES activity in crude forebrain extracts (Fb) and synaptoneurosomes (SN) is given as the FLuc/RLuc ratio. Luciferase activities were measured as described in Materials and Methods. Data are expressed as means \pm SEM of duplicate determinations from three separate experiments. (C) Neuronal FLuc localization. Immunofluorescent localization in 10 DIV neuron-enriched cultures of (i) FLuc (green) and the neuronal marker β -tubulin (red), (ii) FLuc (green) and the neuronal marker MAP2 (red), (iii) FLuc (green) and the glial marker GFAP (red). Fields shown are representative of data obtained from two independent cultures. White arrows indicate typical morphologically characterized astrocytes (bar = 20 μ m).

TABLE 3. FGF-2 IRES activity in primary cultures of cortical neurons or astrocytes

	FLuc/RLuc ratio	
Dissociated cells	E16 embryos, 0.027 ± 0.009	P3–P5 pups, 14.46 ± 0.65
10 DIV cultured cells	Neurons, 1.32 ± 0.29 ^a	Astrocytes, 0.050 ± 0.008 ^a
Fold variation	↑ 49	↓ 289

Cells were dissociated from CTX of RFL12 transgenic mice at E16 or P3–P5, in order to prepare neuron or astrocyte primary cultures, respectively. Cells were grown for 10 DIV. IRES activities were measured in the freshly dissociated cell suspensions and in neurons and astrocytes. Luciferase activities were measured and the FLuc/RLuc ratios were calculated. Values represent the means ± SEM of triplicate determinations obtained from three (astrocyte) or five (neuron) independent cultures.

^a $p < 0.05$.

astrocytes in cocultures. Interestingly, the effect of FGF-2 was not mimicked by another growth factor, brain-derived neurotrophic factor (BDNF), which also promotes neuronal survival (Fig. 7A).

Taken together, these data suggest that FGF-2 is one of the signals released by astrocytes, which contribute to up-regulate FGF-2 IRES activity in neurons.

Regulation of FGF-2 IRES activity by electrical activity and glutamate receptors

Following neuritogenesis, spontaneous electrical activity appears in culture after 1 wk and is involved in neuronal survival, neuronal maturation, and translational activation (Kelleher et al. 2004). Accordingly, we investigated the effect of tetrodotoxin (TTX), a blocker of spontaneous electrical activity on FGF-2 IRES activity. TTX treatment of cortical cocultures from 7 DIV to 10 DIV decreased the FLuc/RLuc ratio by about 40% (Fig. 8).

The blockade of glutamate ionotropic receptors either by D-AP5 (D-2-aminophosphonovalerate), an NMDA (*N*-methyl-D-aspartate) receptor antagonist or by NBQX (6-nitro-7-sulphamoyl-benzo[f]quinoxaline-2,3-dione), an AMPA (*RS*- α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate) receptor antagonist, also decreased the FLuc/RLuc ratio (Fig. 8). Interestingly, a combination of these ionotropic receptor antagonists decreased this ratio to the same extent as TTX. Thus, spontaneous excitatory activity partly contributes to the regulation of FGF-2 IRES activity during development.

DISCUSSION

We report here a specific and stringent spatiotemporal regulation of FGF-2 IRES-dependent translation in the CNS, which takes place into neurons, is dependent upon critical periods of brain development, and is also observed in cell culture during neuronal maturation.

This regulation is specific of the FGF-2 IRES sequence, as other eukaryotic or picornaviral IRESs do not exhibit a similar regulation, and restricted to one tissue, the brain. In addition, cerebral IRES activity is strongly regulated in time and space: during development, levels of IRES activity are very high and peak at the post-natal day P7; in the adult, distribution of IRES activity is heterogeneous within brain areas. Importantly, the integrity of the FGF-2 bicistronic mRNA and the good correlation between Rluc activity and bicistronic mRNA expression clearly demonstrates that the stringent regulation of IRES activity is due to an IRES-

driven mechanism of translation, as documented in other *in vitro* and *in vivo* studies (Creancier et al. 2000; Doerwald et al. 2003; Martineau et al. 2004; Teshima-Kondo et al. 2004; Bonnal et al. 2005; Gonzalez-Herrera et al. 2006). Furthermore, the changes in IRES activity during neuronal maturation and its modulation by electrical activity as well as by pharmacological agents strongly support the physiological relevance of this regulation.

The neuronal localization of IRES activity is demonstrated by its enrichment in synaptoneurosome preparations, by its high activity in neuronal cultures, and by its very low activity in astrocytes. In addition, FLuc protein is always detected by immunocytochemistry into neurons of transgenic mice, but never into astrocytes. Interestingly, increased *N*-myc and APAF1 IRES activities were also reported in neuronal precursors and neuroblastoma cell lines (Jopling and Willis 2001; Mitchell et al. 2003).

A wave of strong increase in IRES activity is observed during the first week of post-natal development at a time of intense synapse formation. As this event should be linked to specific neuronal processes, it may be related to neuronal maturation, which occurs during development or neuronal plasticity, which partly recapitulates this process in the adult. Accordingly, the early maturation of the B&P would explain the early increase of IRES activity at E19 in this structure, whereas the persistent high IRES activity in the adult forebrain and OB would result from the continuous neuronal plasticity described in these brain areas. Interestingly, plasticity in the adult includes translational events (Kelleher et al. 2004) and FGF-2 has been proposed to play a role in this phenomenon (Ishiyama et al. 1991).

The relation between neuronal maturation and FGF-2 IRES-dependent translation is also found in cell culture. The increase of IRES activity measured in neurons correlates with the growth of neuritic processes. In addition, astrocytes that stimulate neuritic outgrowth and participate in synapse formation and stabilization also increase neuronal IRES activity. Finally, we also show that the astrocyte effect is partly due to FGF-2 secretion, which then activates

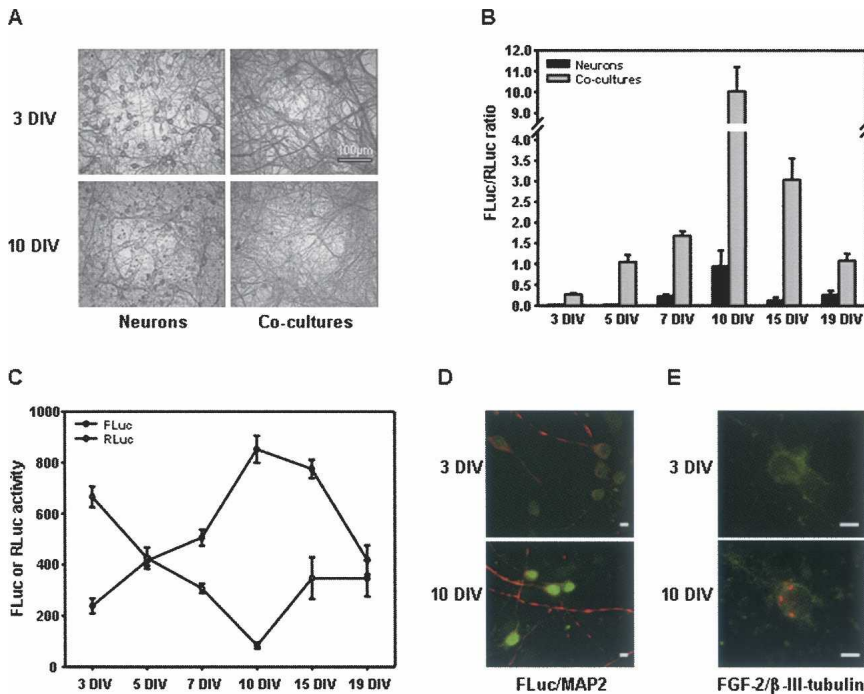


FIGURE 6. Temporal regulation of FGF-2 IRES activity in neuron cultures and neuron/astrocyte cocultures. Cortical cell cultures were prepared as described in Materials and Methods and grown for the indicated time. (A) Development of neuritic processes. Immunolabeling was performed on cultures at 3 and 10 DIV with anti- β -tubulin antibody. Fields shown are representative of data obtained from two independent cultures (bar = 100 μ m). (B) FGF-2 IRES activity during the development of cell cultures. Luciferase activities were measured in neuron-enriched cultures (dark bars) and neuron/astrocyte cocultures (gray bars) from 3 to 19 DIV as described in Materials and Methods. FGF-2 IRES activity is given as the FLuc/RLuc ratio. Data are expressed as means \pm SEM of triplicate determinations from one representative experiment (out of three). (C) Firefly (●) and *Renilla* (○) luciferase activities from 3 to 19 DIV. These data correspond to the values of the ratios shown in B for the neuron/astrocyte cocultures. (D) Fluc expression during the development of the neuron-enriched cultures. Localization of anti-FLuc (green) and anti-MAP2 (red) immunolabeling in 3 and 10 DIV cultures. Fields shown are representative of data obtained from two independent cell cultures (bar = 10 μ m). (E) Endogenous FGF-2 expression during the development of neuron-enriched cultures. Localization of anti-FGF-2 (red) and anti- β -III-tubulin (green) immunolabeling in 3 and 10 DIV cultures. Fields shown are representative of data obtained from two independent cell cultures (bar = 20 μ m).

the neuronal FGF-2 IRES-dependent translation. As FGF-2 has been detected in vivo in a subset of neurons surrounded by astrocytes expressing FGF-2 (Gomez-Pinilla et al. 1994), our data strongly suggest that FGF-2 released by astrocytes stimulates its own synthesis in neighboring neurons through an IRES-dependent mechanism, which in turn provides FGF-2 necessary for neuritic outgrowth and synapse formation.

The changes of IRES activity along neuronal culture are the result of a concomitant but opposite regulation of IRES- and cap-dependent translation and are not linked to variations of bicistronic mRNA expression. This switch in mechanisms, previously described by Dyer et al. (2003), may involve a coordinated regulation of factors specific for each mechanism and/or common factors with opposite

effects on the two translational mechanisms. For instance, one of the specific factors could be hnRNP A1, which may be recruited for FGF-2 IRES activity (Bonnal et al. 2005) and is highly expressed in brain, preferentially in neurons (Faura et al. 1995; Kamma et al. 1995). On the other hand, events linked to cap-dependent translational inhibition such as eIF4E and eIF4E-binding protein (4E-BP) dephosphorylations, eIF2 α phosphorylation, and 4E-BP overexpression have all been associated with IRES-mediated translation (Fernandez et al. 2002; Dyer et al. 2003). Whereas such a switch in translational mechanisms in mammalian cells has often been found associated with stress conditions (Fernandez et al. 2002; Holcik and Sonenberg 2005), we show here that it can also exist under physiological conditions, namely, during neuronal maturation.

In developing synapses, NMDA-receptor activation concomitantly inhibits total protein synthesis and stimulates the translation of α -Ca²⁺/calmodulin-dependent kinase II (α -CaMKII) mRNA (Scheetz et al. 2000), which contains an IRES (Pinkstaff et al. 2001). Similarly, electrical activity and glutamate receptors participate in the stimulation of FGF-2 IRES activity during neuronal maturation. Thus, NMDA receptor activation and, more generally, neuronal inputs may be one of the signals triggering IRES-dependent translation of a subset of mRNAs in neurons. Interestingly, several dendritically localized mRNAs contain an IRES in their 5' UTR (Pinkstaff et al. 2001) and an electrical stimulus is involved in the IRES-dependent translation of the egg-laying hormone in *Aplysia* (Dyer et al. 2003).

Importantly, FGF-2 IRES activity correlates with endogenous expression of the growth factor in neuronal cultures, and its punctuated cytoplasmic signal corresponds to a localization mainly described in neurons of rat brain (Janet et al. 1987; Gonzalez et al. 1995; Fior-Chadi et al. 2007) and retina (Walsh et al. 2001). However, there is a partial overlap between in vivo IRES activity and the endogenous sites of FGF-2 protein expression (Janet et al. 1987; Gomez-Pinilla et al. 1994; Gonzalez et al. 1995). Many reasons can be invoked, notably the alternative cap-dependent translation of FGF-2, but also a differential regulation occurring at the promoter level or in the 3' UTR. It is nevertheless

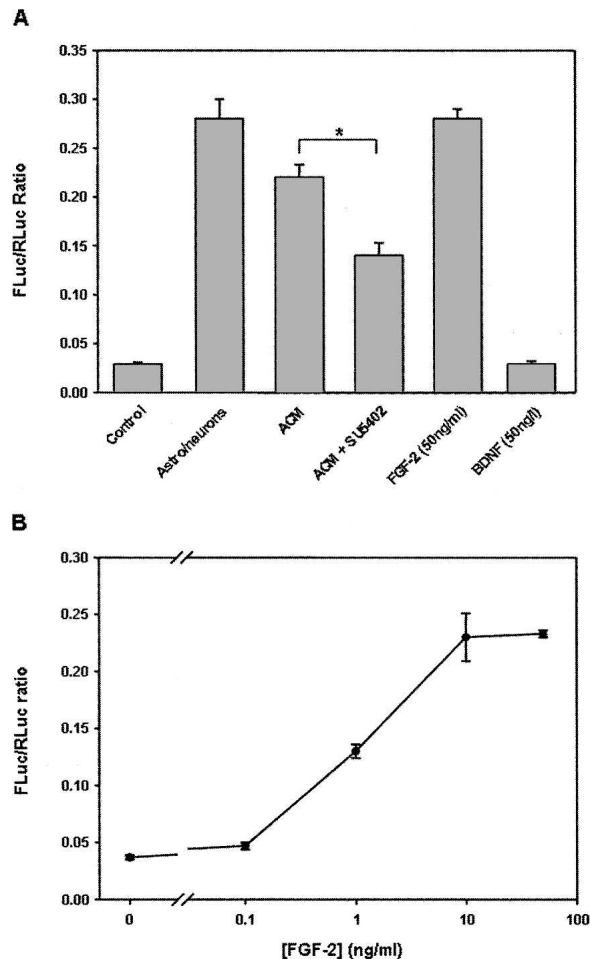


FIGURE 7. Regulation of FGF-2 IRES activity by diffusible factors in 3 DIV neuronal cultures. Neuron-enriched cultures and neuron/astrocyte cocultures were prepared as described in Materials and Methods. Cells were treated 2 h after plating with (A) an astrocyte-conditioned medium (ACM) either in the presence or the absence of FGF receptor 1 inhibitor SU5402 (10 μ M), with FGF-2 (50 ng/mL) or BDNF (50 ng/mL), or with (B) increasing concentrations of FGF-2. Luciferase activities were measured as described in Materials and Methods. FGF-2 IRES activity is given as a FLuc/RLuc ratio. Data are expressed as means \pm SEM of triplicate determinations obtained in a representative experiment (out of three).

noteworthy that FGF-2 has been clearly detected in regions of high FGF-2 IRES activities, i.e., CTX, STR, and HC (Reuss and von Bohlen und Halbach 2003).

Although there is no doubt about the expression and the role of FGF-2 in brain, the conditions leading to its alternative IRES-dependent mode of translation remain to be clarified. The neuronal inputs, which inhibit cap-dependent translation while allowing the expression of a subset of proteins, represent one answer. Perhaps, IRES-dependent translation of some proteins, such as FGF-2, occurring in specific neurons or even synapses while total protein synthesis is inhibited, is necessary for the formation of the neuronal network and later participates in the

plasticity of this neuronal network. FGF-2 also plays a prominent role in post-lesional or post-stress plasticity, a phenomenon that generally involves the reactivation of some developmental mechanisms. Interestingly, Wei et al. (2000) have observed a rapid increase in FGF-2 immunoreactivity following cerebral ischemia, notably into neurons of frontoparietal cortex and striatum, both regions exhibiting a maximal FGF-2 IRES activity. As global protein synthesis is severely inhibited during cerebral ischemia (Althausen et al. 2001), it is tempting to speculate that IRES-dependent translation is turned on to produce FGF-2. Thus, besides its participation in CNS development and plasticity, the IRES-dependent translation of FGF-2 in neurons may also be relevant to brain pathology.

MATERIALS AND METHODS

Materials

All experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/ECC). Versene, antibiotics, and fetal calf serum (FCS) were from Invitrogen and culture media from Eurobio. Monoclonal mouse anti-GFAP (clone G-A-5), anti-PSD95 (clone 7E3-IB8), anti- β -tubulin (clone TUB2.1), and anti-MAP2 (2a+2b) (clone AP-20) were from Sigma, mouse anti-FGF-2 from UBI, and rabbit anti-FLuc from Europa Bioproducts. Rabbit polyclonal anti- β -III-tubulin was from Chemicon (AB9324). Secondary antibodies, i.e., alexa fluor 488-conjugated anti-rabbit, Cy3-conjugated anti-mouse, and

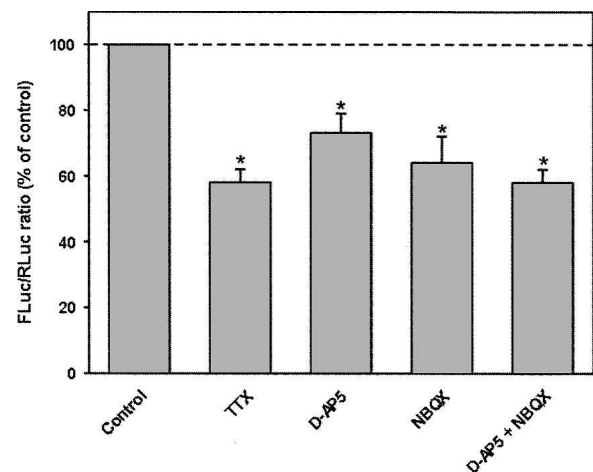


FIGURE 8. Regulation of FGF-2 IRES activity by electrical activity and ionotropic glutamate receptors in 10 DIV neuron/astrocyte cocultures. Cocultures were prepared as described in Materials and Methods. Cells were treated from 7 to 10 DIV with water (control) or TTX (0.5 μ M) or with glutamate receptor antagonists D-AP5 (50 μ M) and NBQX (25 μ M) or a combination of both. Luciferase activities were measured as described in Materials and Methods. FGF-2 IRES activity is given as FLuc/RLuc ratio. Data are expressed as percentages of the FLuc/RLuc ratio obtained in untreated cultures. Values represent the means \pm SEM of triplicate determinations obtained in a representative experiment (out of three).

peroxidase (HRP) conjugated anti-mouse were from Molecular Probes, Jackson Laboratories, and Amersham, respectively. Vectastain ABC Elite and VIP peroxidase substrate kits were from AbCys. SU5402 and Fluorsave were from Calbiochem, TTX from Alomone Labs, and D-AP5 and NBQX from Tocris. “Dual luciferase” kit and “SV total RNA isolation system” were from Promega.

Transgenic mice

Transgenic mice carrying the bicistronic constructs CMV-RLuc-FGF-2 IRES-Fluc, CMV-RLuc-EMCV IRES-Fluc, and CMV-RLuc-c-myc IRES-Fluc and their respective mouse line RFL12, RFLD, RELA, and RMYL-28 are described in Creancier et al. (2000) and Creancier et al. (2001). The transgenic mice carrying the bicistronic constructs CMV-RLuc-VEGF IRES A-Fluc and its mouse line A1 are described in Bornes et al. (2007).

Cortical neuron cultures

The protocol was adapted from Blanc et al. (1999). Cortex from 16-d-old mouse embryos were dissected and incubated for 12 min in Versene. Cells were mechanically dissociated in a defined neuronal culture medium containing DMEM/HAM-F12 supplemented with 33 mM glucose, 2 mM glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 5 mM HEPES, 13 mM sodium bicarbonate, 50 µg/mL transferrin, 87 µM insulin, 1 pM β-estradiol, 3 nM triiodothyronine, 20 nM progesterone, 46 nM sodium selenite, and 100 µM putrescine. Dissociated cells were seeded at a density of 375,000 cells/dish in 24-well plates previously coated with 7.5 µg/mL poly-L-lysine and then with DMEM/HAM-F12 containing 10% FCS, and maintained at 37°C in 5% CO₂ humidified air. For pharmacological treatment, cells cultured in 500 µL neuronal culture medium were incubated with 0.5 µL effector. When necessary, treatments were performed with 0.1% DMSO as the vehicle, a concentration that did not change both luciferase activities. For immunofluorescence, the dissociated cells were seeded on previously coated glass coverslips in eight-well plates at a density of 2×10^6 cells/dish.

Cortical astrocyte cultures

The protocol was adapted from Plachez et al. (2004). Cortex from post-natal 2- to 5-d-old mice were dissected and incubated for 12 min in Versene. Cells were mechanically dissociated and plated in astrocyte culture medium, at a density of 50,000 cells/dish in 24-well plates. Astrocyte culture medium contained DMEM/HAM-F12, supplemented with 33 mM glucose, 2 mM glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 5 mM HEPES, 13 mM sodium bicarbonate, and 10% FCS. Medium was changed every 3 d. Astrocytes reached confluence around 10 DIV.

Neuron/astrocyte cocultures

Astrocyte cultures from wild-type mice were performed as described above except that cells were seeded on dishes previously coated with 7.5 µg/mL poly-L-lysine. After 7 DIV, culture medium was replaced by defined neuronal culture medium. Dissociated cortical cells from E16 transgenic or wild-type embryos were seeded at a density of 375,000 cells/dish on this 70% confluent astrocyte layer. Cultures were then maintained without any medium change.

Preparation of astrocyte-conditioned medium

Cultured cortical astrocytes prepared from wild-type mice as described before were grown for 6 DIV. Culture medium was then replaced by defined neuronal culture medium. Twenty-four hours later, medium was collected and referred to as astrocyte-conditioned medium (ACM). This ACM was then used to replace the medium of cultured neurons, 2h after their plating. In respective controls, medium was replaced by fresh defined neuronal culture medium.

Synaptoneurosome preparation

Synaptoneurosesomes were prepared from forebrains of 6-7-d-old transgenic or wild-type mice according to Recasens et al. (1988).

Measurement of luciferase activities

Renilla and Firefly luciferase activities were measured on lysates as previously described in Creancier et al. (2000) using a “Dual luciferase” kit. Tissues were directly homogenized in lysis buffer with Dounce potter and centrifuged (10 min, 20,000g, 4°C). Cultured cells were washed once in ice-cold phosphate buffered saline (PBS), lysis buffer was added, and plates were stored at –80°C. Cells were then scrapped and lysates were centrifuged. Synaptoneurosomal pellets were directly dispersed in lysis buffer and centrifuged. The supernatants were collected and stored at –80°C. Background values of RLuc and FLuc activities measured in extracts from wild-type mice were subtracted from the values obtained in the corresponding extract from bicistronic transgenic mice. All statistical analyses were calculated using Fisher’s protected least significant difference post hoc test (ANOVA). *P* values 0.05 were considered significant.

Real-time RT-PCR

RNA extraction from RFL12 transgenic mouse tissues or cortical neuron cultures were performed with the “SV total RNA isolation system.” Reverse transcription (RT) was done on 1 to 3 µg total RNA, and real-time PCR was performed with specific probes for RLuc and FLuc as previously described (Teshima-Kondo et al. 2004; Gonzalez-Herrera et al. 2006). Levels of luciferase transcripts were given by the threshold cycle (Ct) value and the data were normalized for the corresponding ribosomal 18S RNA content.

Immunoblotting

Proteins were extracted by sonication in boiling 2% SDS. Samples diluted in loading buffer were boiled for 5 min before loading on a 10% SDS-PAGE. After electrophoresis, proteins were electrotransferred (2 mA/cm²) to nitrocellulose membrane using a semi-dry blotting system. Blots were probed with primary antibodies (1/1000 anti-GFAP or 1/2000 anti-PSD95) and then incubated with HRP-coupled secondary antibody. Bound antibodies were revealed using ECL (Amersham).

Immunocytochemistry

Single labelings were performed directly on plastic wells as described in Blanc et al. (1999). Cells were fixed for 20 min with 4% PF in 0.1 M sodium phosphate buffer and then incubated for 5 min in 8 mM sodium borohydride in PBS. After washes, cells

were preincubated for 1 h in PBS containing 0.2% BSA (PBS-BSA), 10% horse serum, and 0.1% Triton. Incubation with anti- β -tubulin antibody (1/200) was performed overnight at 4°C in PBS-BSA and 1% horse serum. After two washes in PBS-BSA, cells were incubated in biotinylated secondary antibody from the Vectastain ABC Elite kit and samples were processed as described by the manufacturer. HRP activity was revealed using the “VIP peroxidase substrate” kit.

Double-labelings were performed by immunofluorescence on neurons cultured on glass coverslips. Cultures were fixed as described above. After washes, cells were preincubated for 1 h in PBS-BSA containing 20% goat serum and 0.2% Triton. Cultures were then incubated with a combination of two antibodies, anti- β -III-tubulin (1/4000) + anti-FGF-2 (1/250) or anti-FLuc (1/500) + anti-MAP2 (1/500) or anti-FLuc (1/500) + anti- β -tubulin (1/200) or anti-FLuc (1/500) + anti-GFAP (1/500) in PBS-BSA containing 2% goat serum and 0.02% Triton. After three washes in PBS-BSA, cells were incubated with a combination of goat fluorescent secondary antibodies: Alexa fluor 488-conjugated anti-rabbit and Cy3-conjugated anti-mouse antibodies (1/200 and 1/2000 in PBS-BSA, respectively). After three washes in PBS-BSA, coverslips were mounted with Fluorsave and observed on a Leica DMR microscope.

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