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Protein synthesis inhibitors, gene superinduction and memory: Too little or too much protein?

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Abstract

To date, the effects of protein synthesis inhibitors (PSI) in learning and memory processes have been attributed to translational arrest and consequent inhibition of *de novo* protein synthesis. Here we argue that amnesia produced by PSI can be the direct result of their abnormal induction of mRNA—a process termed gene superinduction. This action exerted by PSI involves an abundant and prolonged accumulation of mRNA transcripts of genes that are normally transiently induced. We summarize experimental evidence for the multiple mechanisms and signaling pathways mediating gene superinduction and consider its relevance for PSI-induced amnesia. This mechanistic alternative to protein synthesis inhibition is compared to models of electroconvulsive seizures and fragile × syndrome associated with enhanced mRNA/protein levels and cognitive deficits.

Keywords: Protein synthesis inhibitor; Anisomycin; Cycloheximide; Gene superinduction; Memory; Consolidation; Reconsolidation; Extinction

1. Introduction

Impairments of memory caused by protein synthesis inhibitors (PSI) have served as a basis to posit that memory storage, resulting from consolidation (Davis & Squire, 1984) and reconsolidation (Nader, Schafe, & LeDoux, 2000) processes, critically depends on the synthesis of new proteins in specific brain areas. However, amnesia caused by PSI can be rescued by a variety of hormonal and behavioral manipulations, as discussed in several topical reviews (Gold, 2006; Routtenberg & Rekart, 2005; Squire, 2006). These findings questioned the stand that new protein synthesis is fundamental to memory formation and stimulated alternative ideas on the possible PSI actions. One of them, recently proposed by Gold (2006), suggests that PSI might predominantly exert amnestic effects by introducing meaningless "neuronal noise" to memories. Given the plentiful molecular effects exerted by PSI in different cell systems (Zhelev et al., 1993), this possibility seems likely. Nevertheless, actions other than PSI-induced translational arrest have remained largely unexplored in experimental approaches and theoretical interpretations of PSI actions on neuronal function.

This article will argue that PSI effects on gene superinduction may represent an alternative mechanism by which PSI affect memory processes. Paradoxically, such effects are likely to involve hyperproduction rather than reduction of newly synthesized proteins thereby testing the view that lack of protein synthesis is the underlying mechanism of PSI-induced amnesia.

2. PSI-induced gene superinduction

In the presence of growth factors or other stimulating agents, PSI trigger an abundant accumulation of specific gene transcripts. This phenomenon, known as gene superinduction (Cochran, Reffel, & Stiles, 1983; Lau & Nathans, 1987), is characterized by augmented and prolonged expression of immediate early genes that are typically induced only transiently. In specific cell types anisomycin, one of the most commonly used PSI in memory studies,

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has proved to be particularly potent (when compared to cycloheximide, puromycin and emetine) as an inducer of gene expression (Edwards & Mahadevan, 1992). Several suggested mechanisms contributing to PSI-induced gene superinduction encompass increased mRNA stability (Fort et al., 1987; Rahmsdorf et al., 1987), augmented gene transcription (Greenberg, Hermanowski, & Ziff, 1986), decreased synthesis of labile gene repressors (Wall et al., 1986), and stimulation of nuclear signaling responses (Mahadevan & Edwards, 1991). Depending on the particular mRNA, one or more mechanisms may contribute to gene superinduction.

The degradation of several superinduced mRNAs requires their ongoing translation (Brawerman, 1989; Fort et al., 1987; Wilson & Treisman, 1988), suggesting that protein hyperproduction follows gene superinduction. Although most studies performed to date rarely extend to the analyses of protein translation following gene superinduction, some evidence provides support for delayed protein build up in response to PSI. For example, injection of cycloheximide combined with osmotic shock as a co-stimulus leads to massive accumulation of Fos-like immunoreactivity in dispersed chromatin regions within neurons of the supraoptic nucleus (Lafarga et al., 1993). Following up on their study with cycloheximide-induced memory impairments (Stiedl, Palve, Radulovic, Birkenfeld, & Spiess, 1999), Stiedl and collaborators attempted to determine the cFos levels in mice treated with the PSI before training and then tested 24 h later, anticipating a decrease of cFos in the amnestic animals. Instead, immunohistochemical analyses revealed a massive accumulation of cFos protein in cycloheximide-treated mice (Oliver Stiedl, personal communication, but see Bekinschtein et al., 2007). Notably, *c-fos*, or other PSI-affected genes and the signaling pathways leading to their superinduction (discussed below in more detail) have been strongly implicated in neuronal and synaptic plasticity underlying learning and memory, suggesting that protein overproduction may surpass the requirements for specific synaptic alterations.

3. Characteristics of PSI-induced gene superinduction

It was formerly assumed that gene superinduction arises as a direct or indirect consequence of PSI-induced translational arrest (Kyriakis et al., 1994; Subramaniam, Schmidt, Crutchfield, & Getz, 1989). Meanwhile, several lines of evidence have been generated to suggest otherwise, namely that gene superinduction and protein synthesis inhibition reflect independent actions of PSI.

3.1. Dose requirements

It was demonstrated, using mouse fibroblasts, that anisomycin superinduces the *c-fos* and *c-jun* genes at much lower doses than required for protein synthesis inhibition (Mahadevan & Edwards, 1991). This finding suggested that PSI-induced gene superinduction and translational arrest are dissociable, independent effects of PSI.

3.2. Time-scale of action

Whereas maximal decrease of protein synthesis resulting from PSI-induced translational arrest occurs within 1–2 h postinjection (Flood, Rosenzweig, Bennett, & Orme, 1973), gene superinduction characteristically extends beyond several hours after PSI application (Fort et al., 1987; Greenberg et al., 1986; Wilson & Treisman, 1988). Thus, the consequences of gene superinduction are likely to outlast those of protein inhibition and thereby dominate the final treatment outcome.

3.3. Desensitization

In mammalian cells, pretreatment with anisomycin induces homologous desensitization of intracellular signaling and expression of several genes (*c-fos*, *fosB*, *c-jun*, *junB* and *junD*) while leaving their expression patterns in response to growth factors completely intact (Hazzalin, Le Panse, Cano, & Mahadevan, 1998). Based on these findings, it was suggested that anisomycin acts like a specific signaling agonist (Hazzalin et al., 1998). The binding site mediating these effects is yet to be identified.

3.4. Specificity

PSI-induced superinduction shows not only stimulus specificity, as revealed by the desensitization findings presented above, but also cell type and gene specificity (Table 1). Thus, PSI treatments typically superinduce a subset of genes that may vary among different cell types (Greenberg et al., 1986; Kress & Greenlee, 1997). In the brain, such variations have been observed at a regional level. For example, cycloheximide treatment triggers *Arc* mRNA superinduction in the cortex and some hipocampal areas and a decrease in dentate granule cells (Wallace, Lyford, Worley, & Steward, 1998). It appears therefore that the response to PSI is highly regulated within specific cell types.

3.5. Requirements

In order to obtain maximal effects, PSI-induced gene superinduction typically (but not always) requires co-stimulation by specific growth factors, such as nerve growth factor for the PC12 cell line and fibroblast growth factor for mouse fibroblast cell lines, or less specific co-treatments with phorbol esters, UV irradiation or osmotic shock (Hazzalin et al., 1998). On the other hand, a requirement for co-stimulation in PSI-induced translational arrest has not been reported.

4. PSI-affected genes and signaling pathways

A number of immediate early genes are superinduced by PSI, as listed in Table 1. Preceding gene superinduction, PSI activate distinctive signaling pathways mediating this effect. It is of particular interest that many of the PSI-induced genes and signaling pathways have been implicated in the

Table 1
PSI-induced signaling and gene superinduction in different cell types

Tissue/cell line	Inhibitor (co-stimulation)	Signaling pathway	Super-induced Gene	References
MCAS	Anisomycin	ERK1/2 (not p38 MAPK)	*annexin V*	Konishi, Sato, and Tanaka (2004)
HeLa tk	Anisomycin	Elk-1	*c-fos* (*low and high doses*)	Zinck et al. (1995)
HeLa tk	Cycloheximide	n/a	*c-fos* (*high doses*)	Zinck et al. (1995)
MCG10A cultures	Anisomycin/cycloheximide/ puromycin (TCDD)	AhR	*CYP1A1*	Joiakim, Mathieu, Elliott, and Reiners (2004)
MCG10A cultures	Cycloheximide (TCDD)	AhR, p38, JNK1, JNK2	*CYP1A2 & NMO1*	Joiakim et al. (2004)
Guinea-pig endometrial cells	Cycloheximide (estrogen)	estrogen receptor	*c-fos*	Pellerin et al. (1992)
C3H 10T1/2 mouse fibroblasts	Anisomycin (EGF)	p38 MAPK	*c-fos c-jun*	Cano, Hazzalin, and Mahadevan (1994)
	Puromycin	n/a	*No effect*	Cano et al. (1994)
hepa 1c1c7 mouse hepatoma cells	Cycloheximide (TCDD)	n/a	*cytochrome P_1-450*	Israel, Estolano, Galeazzi, and Whitlock (1985)
C3H 10T1/2 mouse fibroblasts	Anisomycin/Cycloheximide (EGF or TPA)	H3 (correlates with IEG activation)	*c-fos, c-jun*	Mahadevan and Edwards (1991)
A549 epithelial cells	Cycloheximide/actinomycin-D (PMA, IL-1beta, TNFalpha)	n/a	*NFk-B* (*not transcription factors Oct-1, AP-1, Sp-1*)	(Newton, Adcock, and Barnes (1996)
A549 cells	Cycloheximide (proinflammatory cytokines)	NF-kB, JNK	*Cox-2*	Newton et al. (1997)
PC12	Anisomycin (NGF)	p38, JNK, ERK	*c-jun, c-fos,zif268*	Torocsik and Szeberenyi (2000)
EL4.I; EL4.R; BW5147	Cycloheximide (PMA)	n/a	*IL-2*	Zubiaga, Munoz, and Huber (1991)
Various human malignant cell lines	Cycloheximide	n/a	*HuIFN-beta*	Inoue et al. (1991)
Edible snail	Cycloheximide (learning)	n/a	*c-fos*	Grinkevich, Nagibneva, and Lisachev (1997)
3T3 cells	Anisomycin (NGF)	n/a	*c-fos, c-myc actin*	Greenberg et al. (1986)
PC12 cells	Anisomycin (NGF)	n/a	*c-fos, not c-myc*	Greenberg et al. (1986)
C3H 10T1/2 cells	Anisomycin	p38 MAPK	*c-fos, c-jun, fosB, junB, junD*	Hazzalin, Cuenda, Cano, Cohen, and Mahadevan (1997)
Rat brain	Cycloheximide (ECS)	n/a	*Arc, Cox-2, zif268* (*NGFI-A*),	Wallace et al. (1998)

Abbreviations: Arc, activity-regulated cytoskeleton-associated protein; AhR, aryl hydrocarbon receptor; Cox-2, cyclooxygenase-2; CYP1A1, cytochrome p450; ERK, extracellular signal-regulated kinase; EGF, epidermal growth factor; H3, histone 3; *HuIFN-beta*, human interferon-beta; IL-2, interleukin 2; JNK, c-Jun NH(2)-terminal kinase; MCAS, human ovary mucinous cystadenocarcinoma NMO1, NAD(P)H:quinone oxidoreductase; NGF, nerve growth factor; p38 MAPK, p38 mitogen-activated protein kinase, PMA, phorbol myristate acetate; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

formation of long-term memory. Of the listed genes, a significant contribution in memory consolidation has been found for the protein products of *c-fos* (Guzowski, 2002), *c-jun* (Platenik, Kuramoto, & Yoneda, 2000), *CYP1A1* (Kravitz, Meyer, Seeman, Greendale, & Sowers, 2006), *Cox-2* (Melnikova et al., 2006), *zif268*, (Davis, Walker, & Myers, 2003), *IL-2* (Petitto, McNamara, Gendreau, Huang, & Jackson, 1999) and *actin* (Fischer, Sananbenesi, Schrick, Spiess, & Radulovic, 2004) genes. Although memory studies predominantly employed loss-of-function pharmacological and genetic manipulations, superinduction would rather indicate a dysregulated pattern of the expression of a particular gene exceeding the constraints for specific actions required for memory processes.

5. PSI-induced synaptic alterations

The effects of PSI on morphological changes of neurons have not been studied extensively, and the existing data are somewhat variable most likely due to differences in experimental conditions. In cultured Aplysia sensorimotor synapses anisomycin did not block the formation of functional synapses within 1 h after cell contact (Coulson & Klein, 1997) but prevented neurotransmitter-induced changes of varicosities of sensory neurons 24 h posttreatment (Bailey, Montarolo, Chen, Kandel, & Schacher, 1992). Similarly, in rat pyramidal neurons of acute hippocampal slices spinogenesis induced by activation of glucocorticoid receptors was not affected by cycloheximide (Komatsuzaki et al., 2005), however the same PSI prevented spontaneous spine growth up to 1 h after application but not at later time points (Johnson & Ouimet, 2004).

The only study performed to date *in vivo* employed tetanic stimulation of the entorhinal cortex, a procedure resulting in significant enlargement of the dendritic spine area and perimeter of the dentate molecular layer of the hippocampus (Fifkova, Anderson, Young, & Van Harreveld, 1982). Anisomycin pre-treatment blocked this effect when tested 4 min poststimulation. Interestingly, 90 min poststimulation, when anisomycin effects on protein synthesis

inhibition were expected to decay, spine enlargement not only reappeared but showed a significant enhancement when compared to stimulated hippocampi without PSI treatment. Supporting a PSI-induced superinduction mechanism, abundance and elongation of spines has been also associated with increased protein synthesis rates and synaptic protein levels in models of fragil × syndrome (Irwin et al., 2001; Qin, Kang, Burlin, Jiang, & Smith, 2005). In the latter model elevated protein levels are suggested to contribute to long-term depression (LTD) without further need for *de novo* protein synthesis (Nosyreva & Huber, 2006). Analogously, anisomycin produces late phase LTD in cortical slices (Xiong et al., 2006), a finding that seems more consistent with hyperproduction than reduction of protein levels. It is important to note that despite the increased protein synthesis rate and spine abundance, the cognitive consequences in both models are reflected in significant impairments of memory (Davis & Squire, 1984; Zhao et al., 2005) that can be rescued by neurotransmitters (Martinez, Jensen, & McGaugh, 1981; Ventura, Pascucci, Catania, Musumeci, & Puglisi-Allegra, 2004).

6. Implications of PSI-induced gene superinduction for memory

The delayed molecular and structural alterations of neurons based on gene superinduction, suggest that PSI-induced amnesia may originate in the hyperproduction of specific proteins rather than inhibition of global protein synthesis. Acting through this mechanism, PSI might trigger a random or erratic formation of neuronal connections that lack input/output specificity. This possibility may explain several findings.

First, randomness of the effects may result in the formation of correct as well as incorrect synaptic connections, thereby the high variability in the degree, time course, duration and susceptibility to recovery of PSI-induced impairments of memory consolidation (Davis & Squire, 1984) and reconsolidation (Rudy, Biedenkapp, Moineau, & Bolding, 2006). The probability of forming the correct memory however, may increase by manipulations providing enhanced input specificity such as increasing the intensity of stimuli employed to trigger memory consolidation (Flood, Bennett, Orme, Rosenzweig, & Jarvik, 1978) or exposing to reinforced trials during memory reactivation (Cammarota, Bevilaqua, Medina, & Izquierdo, 2004; Fischer et al., 2004). Similarly, hormonal manipulations leading to increased stimulation of monoaminergic receptors (Gold & Sternberg, 1978; McGaugh & Roozendaal, 2002) may increase synaptic weights that favor the formation of a particular memory, and thus rescue PSI-induced memory deficits. In other cases, when similar prior input has already been provided under PSI-free conditions, the probability of forming new but related memories may increase leaving processes such as extinction and latent inhibition intact (Fischer et al., 2004; Lattal & Abel, 2001; Lattal & Abel, 2004; Lewis & Gould, 2004; Mierzejewski et al., 2006; Morris et al., 2006). If extinction trials are performed however under conditions that sufficiently differ from those during training, for example by employing longer exposures (Pedreira & Maldonado, 2003; Power, Berlau, McGaugh, & Steward, 2006), amnestic effects are also observed on extinction learning (Myers & Davis, 2002). The importance of prior neuronal history in susceptibility to PSI effects on log-term plasticity was discussed in more detail recently (Lattal, Radulovic, & Lukowiak, 2006).

Second, memory consolidation involves several interconnected waves of expression of multiple genes (Rampon et al., 2000). Considering that PSI effects on protein synthesis inhibition do not depend on training (Flood et al., 1973; Parsons, Gafford, Baruch, Riedner, & Helmstetter, 2006), it would be expected that PSI would be amnestic over a longer and more continuous posttraining period than it has been observed so far (reviewed by Davis & Squire, 1984; Gold, 2006)). In most cases, however, the strongest PSI effects are observed when the drugs are applied before or shortly after training (Rudy et al., 2006). Intriguingly, the effects of PSI on gene superinduction are the strongest when the drugs are applied under co-stimulation conditions, as can be provided by training or memory reactivation, but not when such manipulations are remote or omitted (Nader et al., 2000; Stiedl et al., 1999; Vianna, Szapiro, McGaugh, Medina, & Izquierdo, 2001). Thus, active neuronal circuits are more likely to be sensitive to PSI-induced gene superinduction than protein synthesis inhibition.

Third, the structural effects of gene superinduction are likely to outlast those of protein synthesis inhibition, and may thereby exert stronger impact on the final PSI treatment outcome. On this basis, instead of attenuating spine formation, an end effect of PSI may involve the formation of large but erratic, malformed and dysfunctional dendritic spines as has been observed in models of fragile × syndrome (Qin et al., 2005) and models of limbic seizures not accompanied by degenerative processes (Bundman, Pico, & Gall, 1994; Leite et al., 2005).

Fourth, electroconvulsive seizures, another commonly used amnestic intervention showing similar memory impairments to those observed by PSI treatment, leads to gene superinduction without inhibiting *de novo* protein synthesis (Altar et al., 2004; Wallace et al., 1998). In fact, a prolonged mRNA expression has been observed in a regional and gene-specific manner after co-application of seizures and cycloheximide (Wallace et al., 1998). These data suggest that the regional superinduction of selected genes may represent a convergent mechanism by which seizures and PSI produce their amnestic effects.

7. Conclusion

Based on vast supporting evidence, the inhibitory effects of PSI on memory formation have been well documented. It remains unclear however, whether the formation of memories depends on *de novo* protein synthesis, the key

anticipated effect of PSI or their other cellular actions. The argumentation supporting PSI-induced gene superinduction as an underlying mechanism of PSI-induced amnesia does not exclude the possibility that protein synthesis, including compartmentalized mRNA translation (Govindarajan, Kelleher, & Tonegawa, 2006), contributes to memory formation. It does however question the view that protein synthesis dependence of memories is verifiable by employing PSI. At this time, it remains unclear to what extent gene superinduction and protein synthesis inhibition contribute individually or combined to PSI-induced amnesia. Rather than extensively employing PSI in memory studies, this issue might be better resolved by developing and applying tools for specific translational arrest of activity-induced mRNAs. Meanwhile, the understanding of the molecular and structural consequences of gene superinduction and protein hyperproduction may prove useful for generating more insight in the mechanisms underlying memory impairments associated with epileptic seizures, fragile × syndrome and possibly other pathophysiological conditions.

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