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Effects of *Gelsemium sempervirens* L. on pathway-focused gene expression profiling in neuronal cells

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ABSTRACT

Ethnopharmacological relevance: *Gelsemium sempervirens* L. is a traditional medicinal plant mainly distributed in the southeastern of the United States, employed in phytotheraphy and homeopathy as nervous system relaxant to treat various types of anxiety, pain, headache and other ailments. Although animal models showed its effectiveness, the mechanisms by which it might operate on the nervous system are largely unknown. This study investigated for the first time by a real-time PCR technique (RT-PCR Array) the gene expression of a panel of human neurotransmitter receptors and regulators, involved in neuronal excitatory signaling, on a neurocyte cell line.

Materials and methods: Human SH-SY5Y neuroblastoma cells were exposed for 24 h to *Gelsemium sempervirens* at 2c and 9c dilutions (i.e. 2 and 9-fold centesimal dilutions from mother tincture) and the gene expression profile compared to that of cells treated with control vehicle solutions.

Results: Exposure to the *Gelsemium sempervirens* 2c dilution, containing a nanomolar concentration of active principle gelsemine, induced a down-regulation of most genes of this array. In particular, the treated cells showed a statistically significant decrease of the prokineticin receptor 2, whose ligand is a neuropeptide involved in nociception, anxiety and depression-like behavior.

Conclusions: Overall, the results indicate a negative modulation trend in neuronal excitatory signaling, which can suggest new working hypotheses on the anxiolytic and analgesic action of this plant.

1. Introduction

Plants from the *Gelsemium* genus are remedies with a long history, used in traditional Chinese medicine, phytotheraphy of various western countries, and homeopathy. *Gelsemium sempervirens* (L.) J.St.-Hil (*Gelsemium sempervirens*) is a twining vine native to warm temperate and tropical America. This plant, commonly known as "Yellow jasmine", though it is not related to the Jasmines, grows along the seacoast from Virginia to south of Florida, extending into Mexico and Guatemala. The first medical records were in 1821 from S. Elliot of the Eclectic school, stating that "root and flowers are narcotic, their effluvia may cause stupor, tincture of the root is used for rheumatism in frictions" (Lloyd, 1911). In the US, it has been extensively used as an arterial sedative and febrifuge in various fevers (Dutt et al., 2010b). The plant extract has been also used in the treatment of restlessness, mental irritability, insomnia, headache and convulsions (Valnet, 1992; Peredery and Persinger, 2004; Dutt et al., 2010a). *Gelsemium sempervirens* is one of the most common and widely used homeopathic remedies and its traditional indications reported by "Materia Medica" are for patients having symptoms like headache, neuralgia, stress complaints and anxiety, emotional excitement, fear, etc. (Boericke, 1927). All parts of the plant contain the major active principle gelsemine as well as other strychnine-related alkaloids, such as gelseminine and sempervirine (Schun and Cordell, 1987; Rujjanawate et al., 2003; Dutt et al., 2010b).

Our (Magnani et al., 2010; Bellavite et al., 2012) and other's (Bousta et al., 2001; Meyer et al., 2013) results have shown that very low doses of *Gelsemium sempervirens* have anxiolytic-like properties in rodent behavioral models, but its action at the

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cellular level still needs to be clarified. In order to further investigate the action mechanism(s) of this plant at the level of neuronal cells, we decided to assess a possible modulation of gene expression. For this purpose, we employed an RT-PCR Array, containing a panel of 84 genes, comprising receptors and regulators of neuronal function, which had been previously utilized in other cell types to investigate low-dose effects of neurotropic substances (Zimmer et al., 2011). The SH-SY5Y human neuroblastoma cells are widely employed in neuropharmacology (Donnici et al., 2008) and may represent a suitable model for investigating the cellular and molecular basis of anxiety (Park et al., 2011).

2. Materials and methods

2.1. Reagents

SH-SY5Y cell line was kindly provided by Prof. U. Armato, Department of Life and Reproduction Sciences, University of Verona. DMEM-F12 medium and fetal bovine serum (FBS) were purchased from Lonza (USA). *Gelsemium sempervirens* solutions were produced by Boiron Laboratoires, Lyon (F). RNeasy Mini kit, RNasefree DNase set, RT first strand kit with random examer primers, SYBR Green qPCR Mastermix were obtained from Qiagen (USA). The RT-PCR Array "Neurotransmitter Receptors and Regulators" was purchased from SABioscience (Qiagen Company, USA)

2.2. Plant materials

Gelsemium sempervirens solutions were produced starting from whole hydroalcoholic extract (Mother Tincture, MT), whose gelsemine content was 6.5×10^{-4} M. MT was diluted 100 times in 30% ethanol/distilled water to obtain the 1c dilution. Subsequent serial 100× dilutions up to 8c, each followed by vigorous shaking were then prepared in the same solvent. The control solutions (pure solvent) were prepared by the same process as the drug dilutions, except that the original plant extract (MT) was absent. Before each experiment we prepared 100× dilutions of *Gelsemium sempervirens* and control solutions 1c and 8c, in ultra pure water followed by vigorous mixing, to obtain 2c and 9c dilutions respectively. We chose to test the 2nd centesimal dilution because this was the highest (i.e., least diluted) dose compatible with cell culture conditions, and we also tested the 9th centesimal dilution because this was an ultra-low (i.e., highly diluted) dose that had previously shown activity in both in vitro (Venard et al., 2011) and in vivo (Magnani et al., 2010) laboratory models. The final gelsemine content in cell assay medium treated with *Gelsemium sempervirens* 2c and 9c was 6.5×10^{-9} M and 6.5×10^{-23} M respectively. The final ethanol concentration in both drug-treated and control solvent-treated cultures was 0.03%. Four replicate experiments were carried out under identical conditions.

2.3. Cell culture

Human SH-SY5Y neuroblastoma cells, cultured at a density of 1×10^6 cells in Petri dishes in DMEM-F12 medium supplemented with 2% fetal bovine serum at 37 °C in a humidified 5% CO_2 atmosphere, were exposed for 24 h to test dilutions and controls (10 ml cell culture + 1.1 ml test solution). This time point was chosen on the basis of previous studies in animal models where the treatments were delivered at 24-h intervals (Magnani et al., 2010). The cell viability was assessed by the WST-1 assay (Ishiyama et al., 1996). A total of 20,000 cells per well were seeded in a 96-well microplate in 200 μl of culture medium supplemented with 22 μl of the *Gelsemium sempervirens* or control solutions. The plates were incubated at 37 °C in a 5% CO_2 atmosphere for 24 h, then 1:10 (v/v) pre-warmed WST-1 solution was added to the cells and the plates incubated for 3 h. The optical density (OD) of the samples was measured using a Victor3 multilabel reader (PerkinElmer, Shelton, CT, USA) at 450 nm, and cell metabolic activity was evaluated as the difference between OD at 3 h and OD at T0.

2.4. RT-PCR

RNA extraction was performed with RNeasy Mini kit including two DNA elimination steps to strictly prevent this kind of contamination (RnaseFree DNase set and Genomic DNA elimination treatment). Four replicate experiments were carried out under identical conditions. 1 μg of total RNA for each sample was reverse transcribed with random hexamer primers and the resulting cDNA was amplified in Opticon2 Real-time instrument (MJ Research) following the thermal profile suggested by manufacturer of the RT-Array. We employed the quantitative RT-PCR Array "Neurotransmitter Receptors and Regulators" with the Sybr Green method, based on validated intron-spanning primers for target genes and several housekeeping controls. The quantification cycle (Cq) for each well was calculated applying the same conditions of baseline and threshold across all the PCR-Array plates and the fold changes (FC) in gene expression levels were computed by the DDCq method, assuming the primer efficiencies to be approximately the same based on the slope of the geometric phase of amplification. The Cq values were analyzed in the PCR Array data analysis web portal (http://www.SABiosciences.com/pcrarraydataanalysis.php), which automatically performed the validation of the plate. All the plates met the quality standard parameters, i.e. absence of genomic DNA contamination, identical reverse transcription efficiency and reaction efficiency.

2.5. Statistical analysis

The normalization of raw Cq data was done against GAPDH, which proved to be the most stable gene. Normalized Cq values were calculated using the formula $2^{-(\text{Cq Gene of Interest} - \text{Cq Reference Gene})}$ ($2^{-\text{DCq}}$) for the correctly amplified genes, i.e. those presenting a single peak in the dissociation melt curve. Four different biological replicates were taken into account for calculating the mean FC and for the statistical analysis, which was performed with the SPSS software (version 20). The differences between expression profiles of the whole panel of genes in various treatment conditions was calculated by the Wilcoxon signed-rank test for paired data (treatment versus its respective control). The differences were ranked, and the positive and negative ranks were separately summed and statistically compared using the specific Wilcoxon tables. If the resulting sums were significantly different, the null hypothesis of the absence of treatment effect was rejected. Fold changes comprised in the interval −0.05 to +0.05 were considered to be null. The differences of expression of each gene were evaluated comparing the FC means of quadruplicate assays in the presence and in the absence of *Gelsemium sempervirens*, with using Student's *t*-test adjusted with Bonferroni for multiple assays.

3. Results

Of the 84 genes that were screened, 39 (46%) did not yield a specific amplification signal visible in the melt dissociation curve and presented Cq values higher than 35, and so were considered as negative calls. The remaining 45 genes were expressed; more specifically, 8 genes (10%) were characterized by a high expression level (corresponding to Cq between 20 and 25), 15 genes (18%) were characterized by medium expression (Cq between 26 and 30),

and 22 genes (26%) were characterized by a low expression profile (Cq between 31 and 35).

As shown in Fig. 1a, most considered genes showed negative FC values (blue bars), so the overall effect of the *Gelsemium sempervirens* 2c dilution on the 45 genes appears to be a slight but statistically significant reduction in gene expression. No clear tendency of the same group of genes was shown after treatment with Gelsemium 9c (Fig. 1b).

Considering the mean changes of each specific gene (Fig. 2a), treatment with *Gelsemium sempervirens* 2c seemed to affect particularly the nicotinic cholinergic receptor subunits β4 (CHRNB4, FC = 0.85 ± 0.10) and γ (CHRNG, FC = 0.84 ± 0.10), the dopamine receptor D2 (DRD2, FC = 0.77 ± 0.07), the prokineticin receptor 2 (PROKR2, FC = 0.72 ± 0.08), and the transcription factor PHOX2A (FC = 0.85 ± 0.11). However, among the genes singled out in this way, statistical analysis by Student's pairwise *t*-test, comparing the normalized 2^{-DCq} values for each treatment with those for the controls, detected a statistically different expression profile only for the PROKR2 gene in *Gelsemium sempervirens* 2c treatment (p adjusted < 0.05, Bonferroni post hoc analysis), whose down regulation was nearly 30%. For the *Gelsemium sempervirens* 9c treatment (Fig. 2b) 2 genes were weakly down-regulated (BRS3, FC = 0.79 ± 0.14 and GRPR, FC = 0.71 ± 0.14), and one was up-regulated (TACR1, FC = 1,65 ± 0.31), all in a non-statistically significant way.

Cell viability was not affected by treatments: OD changes in presence of WST were 0.114 ± 0.00950 vs. 0.115 ± 0.0141 units for *Gelsemium sempervirens* 2c vs. control 2c respectively ($n = 12$, n.s.) and 0.118 ± 0.00642 vs. 0.114 ± 0.0134 units for *Gelsemium sempervirens* 9c vs. control 9c respectively ($n = 12$, n.s.). This suggests that the effects at the level of gene expression were not due to unspecific cell toxicity.

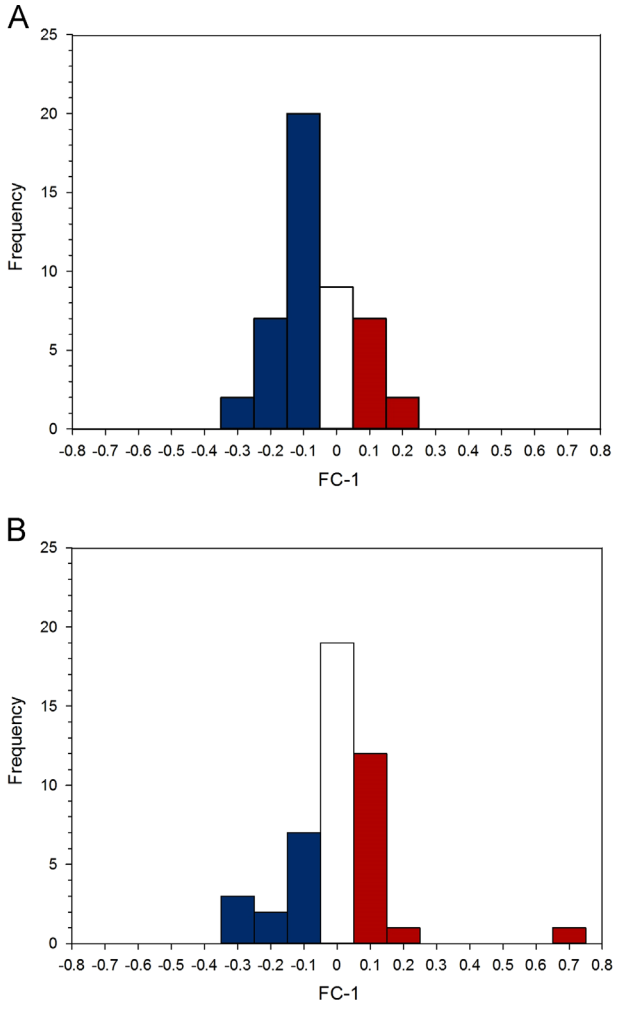

Fig. 1. Frequency of fold change values in the genes of SHSY5Y after *Gelsemium sempervirens* treatments. Mean fold change (FC-1) values from *Gelsemium sempervirens*-treated samples and those from controls were obtained from 4 RT-PCR experiments and their difference was considered as fold change attributable to *Gelsemium sempervirens* effect (see Methods). Only qualitatively- passed amplified genes are reported. Blue bars: frequencies of genes with negative fold change (< -0.05); white bar: frequency of unaffected genes (from -0.05 to 0.05); red bars: frequencies of genes with positive fold change (> 0.05). (A) *Gelsemium* 2c and (B) *Gelsemium* 9c. Data were statistically evaluated by the Wilcoxon signed-rank test for paired data, testing whether the differences are mainly positive or negative, or evenly distributed between the two signs. Wilcoxon statistic (W) p values: (A) < 0.05 and (B) n.s. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

Ethnopharmacology documents a widespread traditional use of *Gelsemium sempervirens*, but knowledge of action mechanisms is still lacking. Recent research has demonstrated, in animal models, the effects of *Gelsemium sempervirens* plant extract and gelsemine on emotional and behavioral responses, highlighting the anxiolytic and antidepressant activity of this drug (Rujjanawate et al., 2003; Dutt et al., 2010a; Magnani et al., 2010; Bellavite et al., 2012; Gahlot et al., 2012; Xu et al., 2012b; Liu et al., 2013; Meyer et al., 2013; Zhang et al., 2013). The major active principles of the *Gelsemium* genus been reported to display sedative, analgesic, and anti-seizure properties (Rujjanawate et al., 2003; Dutt et al., 2010a; Gahlot et al., 2012; Xu et al., 2012b; Liu et al., 2013; Zhang et al., 2013). From the results of the present study a possible suggestion emerges accounting for those observations, involving PROKR2 down-regulation in SH-SY5Y cells. Even if a 30% decrease of expression seems to be not a very striking effect, it is statistically significant and its pharmacological effect cannot be excluded. The down regulation of the PROKR2 gene could be related to a pharmacological activity of *Gelsemium sempervirens* by reducing the effects of endogenous Prokineticin. Prokineticin 1 and 2 (PKs) are newly identified regulatory peptides involved in diverse biological processes ranging from circadian rhythms, inflammatory response, muscle contraction, and nociception to mood disorders (Hu and Qin, 2006). PK2 neuropetide is also involved in the generation of circadian locomotor activity in a behavioral-like manner (Cheng et al., 2002), and in anxiety and depression-related behavior in mice (Li et al., 2009). PK2 triggers activation of the G-protein coupled PK2 receptor, protein kinase C and mitogen activated protein kinase, producing a negative modulation of GABA receptor function (Krishek et al., 1994; Lin et al., 2002; Xiong et al., 2010; Ren et al., 2011). This pathway could be a possible mechanism by which PK2 and PROKR2 increase neuronal excitation, through which this molecular system is involved in anxiety and depression-like behaviors. In our experimental system we identified a novel effect of *Gelsemium sempervirens* at the level of PROKR2 gene expression, which was sufficiently reduced (by 30%) to potentially cause a decreased action of PK2 neuropeptide on synaptic current transmission. Therefore, it is conceivable that a possible effect of *Gelsemium sempervirens* treatment on the nervous system is that it enhances the activity of endogenous GABA by attenuating the PK2-suppressive effect on the phasic response of GABA. This hypothesis is in agreement with the finding that mice lacking the PK2 gene (PK−/− mice) display significantly reduced anxiety in the plus-maze test and show antidepressant-like behaviors in the forced swimming test (Li et al., 2009). This mechanism of *Gelsemium sempervirens* action could be synergistic

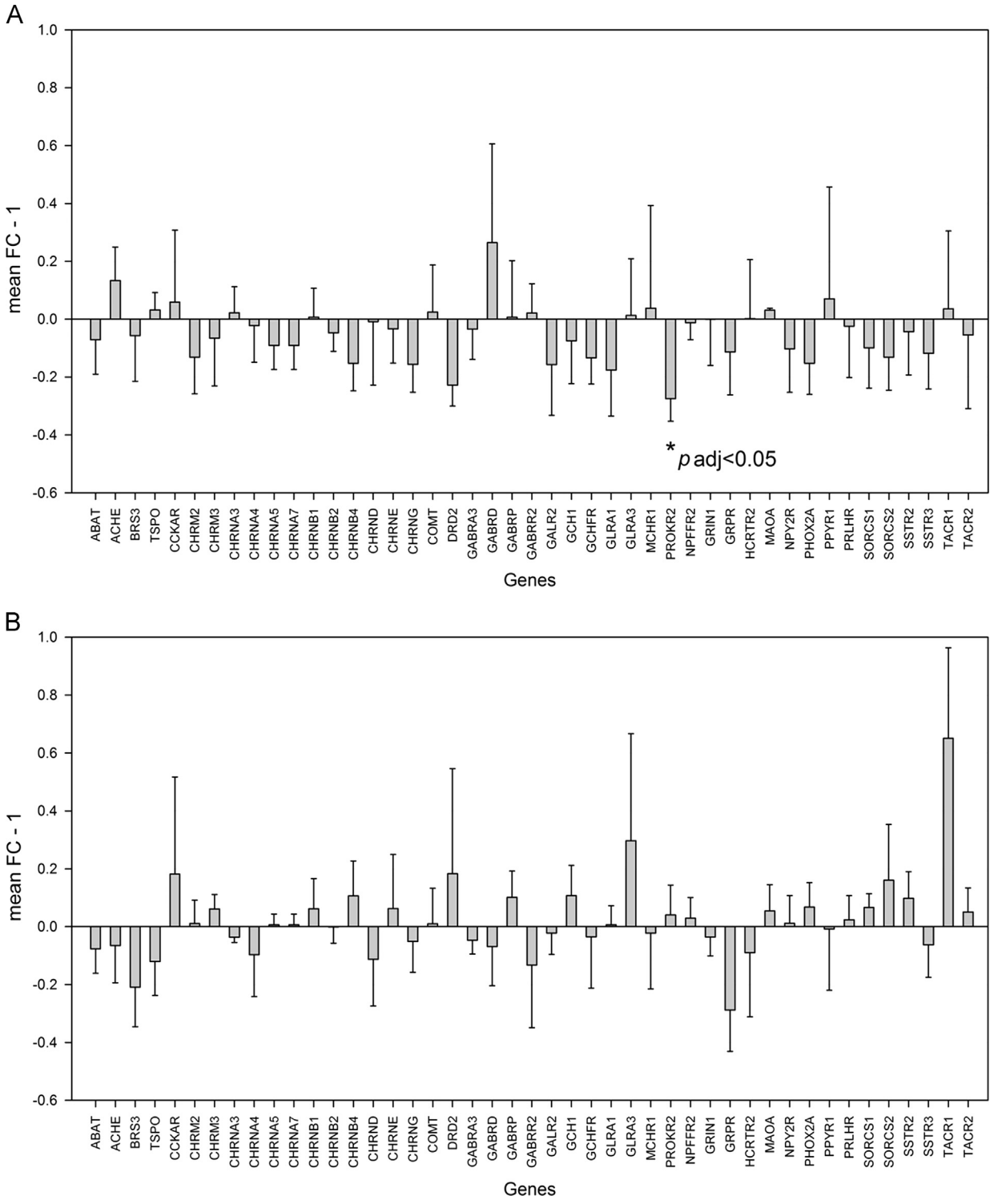

Fig. 2. Effect of *Gelsemium sempervirens* on neurotransmission-focused gene expression in SHSY5Y neurocytes. Values are mean ± SEM of fold changes obtained from 4 different biological replicates and normalized against GAPDH for *Gelsemium sempervirens* 2c treatment (A) and *Gelsemium sempervirens* 9c treatment (B). Data are presented as FC-1 transformed values of qualitatively- passed amplified genes. The Gene symbols in the *X*-axis are from international nomenclature (UniGene, Genbank), as reported in the Quiagen RT-Array (see Reagents). Mean FC-1 values of the effects of *Gelsemium sempervirens* on each gene were statistically evaluated with Bonferroni-adjusted paired Student *t*-test.

with the reported increase in neurosteroids after *Gelsemium sempervirens* stimulation (Venard et al., 2011), which are released locally from neurons, prolonging the decay of such responses and thereby enhancing synaptic inhibition (Belelli and Lambert, 2005).

Given the variety of *Gelsemium* genus effects (Liu et al., 2011; Xu et al., 2012a) and the multiplicity of its alkaloids (Bhattacharyya et al., 2008), it is conceivable that the picture of *Gelsemium sempervirens* action is much more complex and could involve modulation of further pathways. These aspects can be addressed using "omic" approaches such as microarrays and proteomic analysis, which are under investigation in our laboratory.

We also observed a trend toward down-regulation for the DRD2, CHRN4B, CHRNG and PHOX2A (2c) and BRS3, GRPR (9c) genes, suggesting other mechanisms of action of *Gelsemium*

sempervirens at the transcriptional level. For example, the diminished expression of dopamine receptor DRD2 (FC 0.77+0.07) which emerged in our experimental conditions is in keeping with a putative anxiolytic-like effect, since D2-like receptors are considered as inhibitory in GABA and glycinergic neurotransmission (Dyavanapalli et al., 2013). However, since those effects were not corroborated by statistical analysis, the involvement of those genes remains to be clarified. In conclusion, this study surveyed for the first time a panel of neuronal receptors in the SH-SY5Y cells with real-time RT-Array PCR. With this technique a significant down-regulation of PROKR2 gene expression by a nanomolar dilution of *Gelsemium sempervirens* extract was detected, leading to new working hypotheses on the anxiolytic and analgesic action of this plant.

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